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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/47, 7/03, A61K 38/17, 38/43, 38/08, G01N 33/53

A2

(11) International Publication Number: WO 95/26983

(43) International Publication Date: 12 October 1995 (12.10.95)

(21) International Application Number: PCT/US95/03945

(22) International Filing Date: 30 March 1995 (30.03.95)

(30) Priority Data:

08/221,642	31 March 1994 (31.03.94)	US
08/251,691	31 May 1994 (31.05.94)	US
08/291,591	15 August 1994 (15.08.94)	US

(60) Parent Applications or Grants
(63) Related by Continuation

US	08/221,642 (CIP)
Filed on	31 March 1994 (31.03.94)
US	08/251,691 (CIP)
Filed on	31 May 1994 (31.05.94)
US	08/291,591 (CIP)
Filed on	15 August 1994 (15.08.94)

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: TREATMENT OR DIAGNOSIS OF ABNORMAL SIGNAL TRANSDUCTION DISORDERS

(57) Abstract

A method for treatment of a disorder in an organism characterized by abnormal cellular signal transduction is described. The disorder may be characterized by an abnormal level of interaction between a signalling component such as SHC and an RTK of the Trk family, or the interaction between particular SH3 and MP domains (such as an interaction between an SH3 domain and a DYN domain). The method includes disrupting or promoting that interaction (or signal) *in vivo*. The method also involves inhibiting the activity of the complex formed between the individual molecules. A method for diagnosis of such a disease or condition by detecting the level of such interaction as an indication of that disease or condition is also described. Also, a method for screening for an agent useful for treatment of such a disease or condition by assaying potential agents for the ability to disrupt or promote that interaction is described. The invention also features peptides consisting essentially of particular MP, DYN and SH3 domains.

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DESCRIPTION

TREATMENT OR DIAGNOSIS OF ABNORMAL SIGNAL TRANSDUCTION DISORDERS

Related Applications

The present application is a continuation-in-part application of Serial No. 08/221,642, filed March 31, 1994, Serial No. 08/251,691, filed May 31, 1994, and Serial No. 08/291,591, filed August 15, 1994 all of which are incorporated herein by reference in their entirety.

Field of the Invention

The present invention relates generally to the field of celluar signal transduction and more specifically to the diagnosis and treatment of various diseases and conditions associated with abnormal cellular signal transduction pathways.

Background of the Invention

The following is a discussion of relevant art, none of which is admitted to be prior art to the invention.

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of tyrosine residues on proteins. The phosphorylation state of a protein is modified through the reciprocal actions of tyrosine kinases (TKs) and tyrosine phosphatases (TPs).

Receptor tyrosine kinases (RTKs) belong to a family of transmembrane proteins and have been implicated in cellular signaling pathways. The predominant biological activity of some RTKs is the stimulation of cell growth and proliferation, while other RTKs are involved in arresting growth and promoting differentiation. In some instances, a single tyrosine kinase can inhibit, or stimulate, cell proliferation depending on

the cellular environment in which it is expressed. (Schlessinger, J. and Ullrich, A., *Neuron, 9(3)*:383-391, 1992.)

RTKs are composed of at least three domains: an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic catalytic domain that can phosphorylate tyrosine residues. Ligand binding to membrane-bound receptors induces the formation of receptor dimers and allosteric changes that activate the intracellular kinase domains and result in the self-phosphorylation (autophosphorylation and/or transphosphorylation) of the receptor on tyrosine residues. Individual phosphotyrosine residues of the cytoplasmic domains of receptors may serve as specific binding sites that interact with a host of cytoplasmic signaling molecules, thereby activating various signal transduction pathways (Ullrich, A. and Schlessinger, J., 1990, Cell 61:203-212). One family of RTKs is known as the trk family. Barbacid, M. et al., *Biochimica et Biophysica Acta*, 1072:115-127, 1991.

The intracellular, cytoplasmic, non-receptor protein tyrosine kinases do not contain a hydrophobic transmembrane domain or an extracelluar domain and share non-catalytic domains in addition to sharing their catalytic kinase domains. Such non-catalytic domains include the SH2 domains (SRC homology domain 2; Sadowski, I. et al., Mol. Cell. Biol. 6:4396-4408; Koch, C.A. et al., 1991, Science 252:668-674) and SH3 domains (SRC homology domain 3; Mayer, B.J. et al., 1988, Nature 332:269-272). One example of a SH2 domain containing protein is SHC. Pelici, G. et al., Cell 70:92-104, 1992. The non-catalytic domains are thought to be important in the regulation of protein-protein interactions during signal transduction (Pawson, T. and Gish, G., 1992, Cell 71:359-362).

Src-homology 3 (SH3) domains are found in a variety of proteins which are involved in signal trans-

duction or represent components of the cytoskeleton. Recent evidence indicates an involvement of this domain in both negative and positive regulation of cell growth and transformation and an important role in interactions between components of the cytoskeleton. While it has been shown that deletion or mutation of SH3 domains activates the transforming potential of nonreceptor tyrosine kinases, suggesting involvement in negative regulation of an intrinsic transforming activity of such proteins (Jackson and Baltimore, *EMBO J.* 8:449-456, 1989; Hirai and Varmus, *Mol. Cell. Biol.* 10:1307-1318, 1990; Seidel-Dugan et al., *Mol. Cell. Biol.* 12:1835-1845, 1991), it was recently demonstrated that the SH3 domain of the GRB-2 adaptor protein interacts with the exchanger protein son of sevenless (SOS), which links RTKs to the ras system and therefore represents an essential element in the mitogenic signalling pathway.

Specific mutations that impair the ability of GRB-2 and its *C. elegans* homologue, sem-5, to transmit a mitogenic signal or to mediate vulval development, respectively, were localized to the SH3 domain. Lowenstein et al., *Cell* 70:431-442, 1992; Chardin et al. *Science* 260:1138-1143, 1993; Egan et al., *Nature* 363:45-51, 1993; Rozakis-Adcock et al., *Nature* 363:83-85, 1993; Li et al., *Nature* 363:85-88, 1993. Its presence in a variety of cytoskeleton-associated proteins, including spectrin, myosin 1, and an actin-binding protein from yeast, ABP-1, implies that SH3 domains may participate in regulating cytoskeleton integrity and rearrangement. Koch et al., *Science* 252:668-674, 1991; Drubin et al., *Nature* 343:288-290, 1990; Rodaway et al., *Nature* 342:624, 1989. Furthermore, Cicchetti *et al.* (*Science* 257:803-806, 1992) demonstrated binding of the Abl-SH3 domain to 3BP1, a protein with homology to GAP-Rho, suggesting a relationship to rho and rac GTP binding proteins, which have been implicated in the regulation of membrane ruffling

and the assembly of actin filaments. (Ridley et al., *Cell* 70:389-399, 1992; Ridley et al, *Cell* 70:401-410, 1992) Evidence from several laboratories indicates that SH3 binding sites on target proteins involve short proline-rich sequences with an apparent consensus motif. Ren et al., *Science* 259:1157-1161, 1993.

A central feature of signal transduction (for reviews, see Posada, J. and Cooper, J.A., 1992, Mol. Biol. Cell 3:583-392; Hardie, D.G., 1990, Symp. Soc. Exp. Biol. 44:241-255), is the reversible phosphorylation of certain proteins. Receptor phosphorylation stimulates a physical association of the activated receptor with target molecules. Some of the target molecules are in turn phosphorylated. Such phosphorylation transmits a signal to the cytoplasm. Other target molecules are not phosphorylated, but assist in signal transmission by acting as adapter molecules for secondary signal transducer proteins. For example, receptor phosphorylation and the subsequent allosteric changes in the receptor recruit the *Grb*-2/SOS complex to the catalytic domain of the receptor where its proximity to the membrane allows it to activate *ras*. Pawson, T. and Schlessinger, J., Current Biol. 13:434, 1993.

The secondary signal transducer molecules generated by activated receptors result in a signal cascade that regulates cell functions such as cell division or differentiation. Reviews describing intracellular signal transduction include Aaronson, S.A., *Science*, *254*:1146-1153, 1991; Schlessinger, J. *Trends Biochem. Sci.*, *13*:443-447, 1988; and Ullrich, A., and Schlessinger, J., *Cell*, *61*:203-212, 1990.

Dynamin is a GTP-binding protein that has been implicated in microtubule-based motility and endocytosis. Robinson et al., *Nature* 3658:163-166, 1993. ^1H chemical shift changes induced by complex formation with a synthetic peptide derived from the SH3-binding protein dynamin, together with amino acid sequence comparisons,

have been said to suggest that the ligand-binding site consists of a hydrophobic surface flanked by two charged loops. Booke et al., *Cell* 73:813-822, 1993.

Five proline-rich sequences that may be SH3 domain-binding sites within dynamin have been predicted. Gout et al., *Cell* 75:25-36, 1993. The potential variety between SH3 domain-binding motifs was said to require more detailed study that will be complicated in the case of dynamin because of the presence of multiple overlapping potential binding sites. Id. The possibility that the assay didn't reflect the affinities displayed by the molecules could not be excluded. Id. The location of a specific site that might be required for alteration of dynamin GTPase activity had not been located. Id. One of the next important steps was said to be to clearly identify those SH3-containing proteins that are able to interact with dynamin in vivo. While it was said that co-immunoprecipitiation of PLCγ and dynamin had been observed the particular domains involved were not mentioned. Id.

While it is widely appreciated that RTKs assume a key role in signal transduction, the part played by phosphatases is less understood. Like the RTKs, the tyrosine phosphatases comprise a family of transmembrane and cytoplasmic enzymes. See, for example, Hunter, *Cell* 58:1013-1016, 1989, Charbonneau, *Proc. Natl. Acad. Sci.USA* 85:7182-7186, 1988, Jiang, *Mol. Cell Bio.* 13:2942-2951, 1993 and Fischer, E., *Science* 253:401-406, 1991. In all cases, the putative receptor tyrosine phophatase (RTP) ligands have yet to be identified. Some TPs and TKs contain similar structural components. For example, members of both protein families may contain a homologous SH2 domain. For a review, see Koch, *Science* 252:668-674, 1991.

Although RTPs appear to be an integral part of the signal transduction mechanism, in most cases their specific functions have not been defined. Walton, *Ann.*

Rev. Biochem. 62:101-120, 1993. It is believed that RTKs play a triggering role in signal transduction, while RTPs guarantee that the trigger is reset, thereby serving to deactivate the pathway.

The product of the *vav* protooncogene is a hematopoietic cell-specific, signal-transducing protein that displays oncogenic potential upon deletion of N-terminal sequences (Katzav et al., *EMBO J.* 8:2283-2290, 1989; Katzav et al. (1991) *Mol. Cell. Biol.* 11:1912-1920, 1991). The 95 kD vav protein becomes tyrosine-phosphorylated after engagement with a variety of cell surface receptors, including the T-cell receptor (Bustelo et al. (1992) *Nature* 356:68-71, 1992; Margolis et al., *Nature* 356:71-74, 1992), FceR-receptor (Margolis et al., *Nature* 356:71-74, 1992), IgM-receptor (Bustelo and Barbacid *Science* 256:1196-1199, 1992), the *c-kit* gene product $p145^{c\text{-}kit}$ (Alai et al., *J. Biol. Chem.* 267:18021-18025, 1992), the IL2-receptor (Evans et al., *Biochem. J.* 294:339-342, 1993), and the IFNa receptor (Platamias and Sweet, *J. Biol. Chem.* 269:3143-3146, 1994).

Vav structural domains that are thought to play a role in signal transduction include a rho guanine nucleotide release factor (GRF) homology domain (Adams et al., *Oncogene* 7:611-618, 1992), a Pleckstrin homology (PH) domain (Musacchio et al., *TIBS* 18:343-348, 1993), a Cys/His-rich region that resembles phorbol ester binding proteins (Adams et al., *Oncogene* 7:611-618, 1992), one SH2 and two SH3 domains (Pawson and Schlessinger *Curr. Biol.* 3:434-442, 1993).

The vav rhoGRF homology domain has recently been shown to be responsible for the activation of *ras* after T-cell activation (Gulbins et al., *Science* 260:822-825, 1993), while the SH2 domain of vav has been demonstrated to couple to autophosphorylated receptor tyrosine kinases, including receptors that are normally not found in hematopoietic cells types, such as the

epidermal growth factor receptor (Bustelo et al. (1992) *Nature* 356:68-71, 1992; Margolis et al., *Nature* 356:71-74, 1992). The significance of the vav SH3 domains, however, and their interaction targets have remained unknown until now.

Summary of the Invention

This invention relates to products and methods useful for screening, diagnosing, and treating a disorder characterized by an abnormality in a signal transduction pathway, wherein the signal transduction pathway involves the interaction between: (a) a member of the Trk family of RTKs and a signaling component; (b) a heterogeneous ribonucleoprotein MP domain and a SH3 domain; (c) a MP domain and a vav protein SH3 domain; or (d) a SH3 domain and a DYN domain. It has been determined that the particular interactions between the specific molecules and domains listed above are required for differentiation and survival of cells and are also associated with the basic signaling function of proteins associated with various diseases or conditions.

Thus, in a first aspect, the invention features a method for treatment of a disease or condition in an organism characterized by an abnormality in a signal transduction pathway, wherein the signal transduction pathway involves the interaction between: (a) a receptor tyrosine kinase of the Trk family and a signalling component; (b) a heterogenous ribonucleoprotein MP domain and a SH3 domain; (c) a MP domain and a vav protein SH3 domain; or (d) a SH3 domain and a DYN domain. The disorder may also be characterized by an abnormal level of interaction between the individual molecules. The method includes disrupting or promoting that interaction (or signal) in vivo. The method also involves inhibiting or promoting the activity of the complex formed between the individual moleculeslisted above in (a) through (d).

The disease or condition may also be character-

ized by an abnormal level of interaction between the individual molecules. An abnormal interaction level may also either be greater or less than the normal level and may impair the normal performance or function of the organism. Since the interaction between the individual molecules is part of the signal transduction pathway, it is still possible to treat such a disease by interfering with the level of interaction between the individual molecules. Thus, it is also possible to screen for agents that will be useful for treating a disease or condition, characterized by an abnormality in the signal transduction pathway, by testing compounds for their ability to affect the interaction between the individual molecules since the complex formed by such interaction is part of the signal transduction pathway. However, the disease or condition may be characterized by an abnormality in the signal transduction pathway even if the level of interaction between the individual molecules is normal.

Examples of diseases or conditions to be treated or diagnosed by the present invention include Alzheimer's disease, Parkinson's disease, Lou Gerhig's disease (ALS), trauma, damaged or severed nerve injuries, Huntington's chorea, multiple schleroris, muscular dystrophy, syringomiplia, Tabes Dorsalis, and cardiovascular accidents. These and other diseases or conditions are often characterized by one or more of the following symptoms: atasia, aphasia, paralysis, paresea, and paralgies. Examples of other diseases or conditions to be treated or diagnosed by the present invention include leukemia, hematopoietic cell disorders, embryonic disorders, cancers, lymphoproliferative disorders, hyperplasia, fibroses, and hyperproliferative disorders such as psoriasis and neurofibromatosis. These and other diseases or conditions are often characterized by unwanted cell growth which displaces or interferes with normal tissue

organization resulting in a vast array of clinical symptomatology and organ damage, including the formation of tumors.

An abnormal interaction level may also either be greater or less than the normal level and may impair the normal performance or function of the organism. Thus, it is also possible to screen for agents that will be useful for treating a disease or condition, characterized by an abnormality in the signal transduction pathway, by testing compounds for their ability to affect the interaction between the individual molecules, since the complex formed by such interaction is part of the signal transduction pathway. However, the disease or condition may be characterized by an abnormality in the signal transduction pathway even if the level of interaction between the individual molecules is normal.

By "organism" is meant any living creature. The term includes mammals, and specifically humans. One organism of interest is mice, as the ability to treat or diagnose mice is often predictive of the ability to function in other organisms such as humans.

By "interact" is meant any physical association between proteins, whether covalent or non-covalent. Examples of non-covalent bonds include electrostatic bonds, hydrogen bonds, and Van der Waals bonds. Stryer, Biochemistry, 1988, pages 7-8. Furthermore, the interactions between proteins may either be direct or indirect. Thus, the association between two given proteins may be achieved with an intermediary agent, or several such agents, that connects the two proteins of interest. Another example of an indirect interaction is the independent production, stimulation, or inhibition of either of the individual molecules by a regulatory agent. (Schlessinger, 1992, Neuron 9:383-391) Depending upon the type of interaction present, various methods may be used to measure the level of interaction. For example, the strengths of covalent bonds are often measured in

terms of the energy required to break a certain number of bonds (i.e., kcal/mol) Non-covalent interactions are often described as above and also in terms of the distance between the interacting molecules. Indirect interactions may be described in a number of ways, including the number of intermediary agents involved, or the degree of control exercised over one molecule present in the complex relative to the control exercised over the other molecule of the complex.

By "Trk" it is meant any one of the Trk family of tyrosine kinase receptors, including but not limited to TrkA, TrkB and TrkC. Those skilled in the art will recognize other members of this family based on homology of their amino acid and/or DNA sequences. See, for example, Klein, R. et. al., EMBO Journal 8:3701-3709, 1989 and Barbacid, M. et. al. Biochemica et Biophysica Acta, 1072:115, 127, 1990. Some members of the TRK family are activated by phosphorylation of tyrosines and have a cytoplasmic domain with at least one binding site for an SH2 domain containing signalling component.

By "signalling component" is meant a protein that interacts with a receptor of the Trk family. The signalling component may be a SH2 domain containing protein. Examples of signalling components include SHC, phospholipase Cγ (PLCγ), Grb-2, Sos, ras, raf, MAP-Kinase, MAP-Kinase kinase and phosphatidylinositol-3'-kinase (PI3'-K).

By "heterogenous ribonucleoprotein" is meant a macromolecule that contains both RNA and protein. The RNA sequence is preferably about 500 nucleotides long and the protein preferably has a consensus sequence as shown in Alberts et al., Molecular Biology of The Cell, Second Edition, pp. 532-533, 1989, incorporated herein by reference, in its entirety, including any drawings. In preferred embodiments the heterogenous ribonucleoprotein is heterogenous ribonucleoprotein K and is not p62.

8:2283-2290, 1989, incorporated herein by reference in its entirety, including any drawings. Vav proteins include sequences with additions, deletions and/or mutations in the sequence given in Katzav et al., supra, so long as the SH3 domain retains the ability to bind a MP domain. In preferred embodiments the vav protein has the structural motifs as depicted in Margolis et al., *Nature* 356:71-74, 1992, incorporated herein by reference in its entirety, including any drawings, and has a molecular weight of approximately 95 kD.

By "DYN domain" is meant an amino acid sequence which binds an SH3 domain as described by Gout et al., supra, (hereby incorporated herein by reference) e.g., those consensus sequences listed in Table 2 of Gout et al., supra, such as PXXXPPXXP (SEQ. I.D. NO. 1) or PXXPPXXP (SEQ. I.D. NO. 2), and the sequence APPVPSRG (SEQ. I.D. NO. 3), where X is any amino acid. The present invention also features the use of DYN domain containing fragments consisting essentially of 6-12 amino acids in length, such as the PPVPSR motif.

By "disrupt" is meant that the interaction between the individual molecules is reduced either by preventing expression of one molecule, or by specifically preventing interaction of the naturally synthesized proteins or by interfering with the interaction of the proteins.

By "promote" is meant that the interaction between individual molecules is increased either by increasing expression of one molecule, or by decreasing the dephosphorylating activity of the corresponding regulatory TP or other phophatase acting on other phophoralated signalling components by promoting interaction of the individual proteins or by prolonging the duration of the interaction.

By "signal transduction pathway" is meant the sequence of events that involves the transmission of a message from an extracellular protein to the cytoplasm

By "MP domain" is meant a proline rich amino acid sequence which binds an SH3 domain, approximately 5-20 amino acids in length, such as PLPPPPPPRG (SEQ. I.D. NO. 1). Examples of other MP domains include those shown in Table 1 and Figure 5 of Ren et al., *Science* 259:1157-1161, 1993, incorporated herein by reference in its entirety, including any drawings. Other examples of MP domains include the proline-rich sequences shown in Table 2 of Gout et al., *Cell* 75:25-36, 1993, incorporated herein by reference in its entirety, including any drawings. The present invention also features the use of MP domain containing fragments consisting essentially of 8-12 amino acids in length. In preferred embodiments the MP domain is found in heterogenous ribonucleo-proteins such as hnRNP-K.

By "SH3 domain" it is meant an amino acid sequence (preferably 50-75 amino acids, more preferably about 60 amino acids) as described by Musacchio et al., *FEBS* 307(1):55-61, 1992 (incorporated herein by reference, in its entirety, including any drawings), e.g., those sequences listed in Figure 2 of Musacchio et al., supra, such as the consensus sequence listed therein. Those skilled in the art will recognize other SH3 domains based on homology of their amino acid and/or DNA sequences. In preferred embodiments the SH3 domain is not part of GAP, but is a vav protein SH3 domain, specifically the C-terminal SH3 domain of $p95^{vav}$, interacts with a heterogenous ribonucleoprotein MP domain, and binds a DYN domain as described by Musacchio et al., *FEBS* 307(1):55-61, 1992 (incorporated herein by reference) e.g., those sequences listed in Figure 2 of Musacchio et al., supra, such as the consensus sequence listed therein.

By "vav protein" is meant a protein that has a SH3 domain that binds to one or more MP domains, preferably about 800 amino acids long and substantially as depicted in Figure 3 of Katzav et al., *EMBO J.*

through a cell membrane. The signal ultimately will cause the cell to perform a particular function, for example, to proliferate and therefore cause cancer. Various mechanisms for the signal transduction pathway (Fry et al., 1993, Protein Science, 2:1785-1797) provide possible methods for measuring the amount or intensity of a given signal. Depending upon the particular disease associated with the abnormality in a signal transduction pathway, various symptoms may be detected. Those skilled in the art recognize those symptoms that are associated with the various other diseases described herein. Furthermore, since some adapter molecules recruit secondary signal transducer proteins towards the membrane, one measure of signal transduction is the concentration and localization of various proteins and complexes. In addition, conformational changes that are involved in the transmission of a signal may be observed using circular dichroism and fluorescence studies.

In a related aspect the invention features a method for screening for an agent useful for treatment of such a disease or condition by assaying potential agents for the ability to disrupt or promote that interaction. The screening may also involve assaying potential agents for the ability to remove or reduce the effect of an abnormality in a signal transduction pathway, wherein the signal transduction pathway contains the individual molecules listed above.

Useful agents for treatment of such diseases can be identified by standard screening protocols in which measurement of such interaction is determined. For example, such an agent may be a peptide which either comprises, consists of, or consists essentially of a src-homology 2 (SH2) domain of a signaling component or, alternatively, a phosphotyrosine-SH2-domain-binding motif, a DYN domain or particular SH3 or MP domains.

By "screening" is meant investigating an organism for the presence or absence of a property. The

process may include measuring or detecting various properties, including the level of signal transduction and the level of interaction between SHC and Trk, between a SH3 domain and a DYN domain or between a SH3 domain and a MP domain.

This invention also relates to methods for diagnosis and treatment of a disorder, most preferably a disorder characterized by an abnormality in a signal transduction pathway, wherein the signal transduction pathway involves the interaction between: (a) a receptor tyrosine kinase of the Trk family and a signaling component; (b) a heterogenous ribonucleoprotein MP domain and a SH3 domain; (c) a MP domain and a vav protein SH3 domain; or (d) a SH3 domain and a DYN domain. In particular, this invention relates to methods for promoting and/or inhibiting the interaction between the molecules listed above.

By "diagnosis" is meant any method of identifying a symptom normally associated with a given disease or condition. Thus, an initial diagnosis may be conclusively established as correct by the use of additional confirmatory evidence such as the presence of other symptoms. Current classification of various diseases and conditions is constantly changing as more is learned about the mechanisms causing the diseases or conditions. Thus, the detection of an important symptom, such as the detection of an abnormal level of interaction between the individual molecules of the complex (or the detection of an abnormal level of signal transduction in a pathway containing such molecules) may form the basis to define and diagnose a newly named disease or condition. For example, conventional neurological diseases are classified according to the presence of a particular set of symptoms. However, a subset of these symptoms may both be associated with an abnormality in a particular signalling pathway, such as the *ras*[21] pathway and in the future these diseases may be reclassified as

ras^{21} pathway diseases regardless of the particular symptoms observed.

In preferred embodiments, the disease or condition which is diagnosed or treated are those described above, and the agent is a dominant negative mutant protein provided by gene therapy or other equivalent methods as described below. That is, the agent is a peptide which blocks or promotes interaction of the individual molecules. The peptide may be recombinant, purified, or placed in a pharmaceutically acceptable carrier or diluent. In preferred embodiments, the SH3 domain may be a PLCγ or GRB-2 SH3 domain or a vav protein SH3 domain and the MP domain may be a heterogenous ribonucleoprotein MP domain.

By "dominant negative mutant protein" is meant a mutant protein that interferes with the normal signal transduction pathway. The dominant negative mutant protein contains the domain of interest (e.g., an SH3, or MP domain), but has a mutation preventing proper signaling, for example by preventing binding of a second domain from the same protein. One example of a dominant negative protein is described in Millauer, B. et al., Nature February 10, 1994.

In a further related aspect, the invention features a method of identifying the receptor tyrosine phosphatase responsible for dephosphorylating the activated Trk receptor or other individual molecules, thereby regulating the signaling pathway. Novel methods of treatment of disorders (e.g., nuerological disorders) can be based on modulating this phosphatase activity.

Modulation of the RTP activity can be accomplished in a variety of ways including but not limited to the use of compounds or drugs that inhibit or enhance the RTP activity, antisense or ribozyme approaches that "knock out" the RTP activity, or gene therapy approaches to correct defects in the RTP or restore the regulated expression of the RTP. Compounds can be used that

specifically modulate the activity of the controlling RTP, thereby prolonging or enhancing signal transduction mediated by the receptor.

In a further related aspect, the invention features a method of identifying the receptor tyrosine phosphatase responsible for dephosphorylating the activated SH3:DYN (or SH3:MP) receptor, thereby regulating the receptor signaling pathway. By "SH3:DYN receptor" is meant the receptor that binds the preformed complex of proteins that have SH3 and DYN domains, such as dynamin and PLCγ or dynamin and GRB-2. By SH3:MP receptor is meant the receptor that binds the preformed complex of proteins that have SH3 and MP domains. Novel methods of treatment of disorders (e.g., neurological disorders) can be based on modulating this phosphatase activity. Modulation of the RTP activity can be accomplished in a variety of ways including but not limited to the use of compounds or drugs that inhibit or enhance the RTP activity, antisense or ribozyme approaches that "knock out" the RTP activity, or gene therapy approaches to correct defects in the RTP or restore the regulated expression of the RTP. Compounds can be used that specifically modulate the activity of the controlling RTP, thereby prolonging or enhancing signal transduction mediated by the receptor.

In another aspect the invention features a method for screening non-hematopoietic cells for a protein having a SH3 domain that interacts with a heterogenous ribonucleoprotein MP domain. The method involves exposing a protein with a SH3 domain from a non-hematopoietic cell to a heterogenous ribonucleoprotein MP domain and detecting the level of interaction.

In another aspect the invention features peptides comprising, consisting essentially of, or consisting of a particular domain, for example a DYN domain or a particular SH3 or MP domain.

By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

Fig. 1 is a diagrammatic representation of a receptor tyrosine phosphatase modulating the activity of a receptor tyrosine kinase. On the left hand side of the figure, ligand binds to an RTK, which dimerizes and becomes phosphorylated. On the right hand side of the figure, ligand binds to an RTP, which then dephosphorylates the activated RTK thereby modulating signal transduction.

Fig. 2 is a diagrammatic representation of some of the possible multiple signal transduction pathways activated by a single activated receptor tyrosine kinase. PLC-gamma = phospholipase C-gamma; DAG = diacylglycerol; IP3 = inositol 1,4,5-triphosphate; PtdIns-3k = phosphatidylinositol 3-kinase; PtdIns(3)P =

phosphatidylinositol 3-phosphate; GAP = *ras* GTPase-activating protein.

Fig. 3 is a diagrammatic representation of substrate binding capacities of receptor constructs. The cytoplasmic portions of the PDGF-receptor:Trk chimeric mutants (PT-R) are shown to the right of the schematically indicated plasma membrane. Black boxes denote the tyrosine kinase domain. PT-Y3F is an abbreviation for PT-Y490/751/785F, wherein point mutations have been made to replace the tyrosines at the designated positions with a substitute amino acid.

Figure 4 shows a schematic representation of vav primary structure and the constructs used as GST fusion proteins. The SH3 domain of *vav* is the major determinant of its specific association with hnRNP-K.

Neuronal Diseases and Signal Transduction

Differentiation and survival of neuronal cells is mediated, in part, by the activity of a family of related RTKs, including TrkA, TrkB, and TrkC and ligands such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins-3 and 4 (NT-3 and NT-4). Barbacin, M., *Biochimica et Biophysica Acta* 1072:115-127, 1991, Kaplan, D.R., *Science* 252:554-558, 1991; Klein, R., *Cell* 65:189-197, 1991; Davies, A.M., Nature 368:193-194, 1994 and Klein, R., *Cell* 66:395-403, 1991. A number of cellular signal transduction pathways are stimulated upon ligand binding, including those pathways involving phopholipase C-gamma (PLC-gamma), *ras* and the serine-threonine kinase *raf*. Although the biological consequences of neuronal RTK activation are fairly well characterized, the identity of the key cellular components in these processes was incompletely understood. Wood, K.W., *Proc. Natl. Acad. Sci.* 90:5016-5020, 1993 and Obermeier, A., *EMBO J.*, 12(3):933-941, 1993.

Abnormalities in signal transduction pathways

can lead to various diseases through both underactivity and overactivity. Examples of disorders which are characterized by underactivity of a signal transduction pathway include various neurodegenerative diseases such as myasthenia gravis, amyotrophic lateral sclerosis, cervical spondylosis, and Alzheimer's disease. A neurological disease possibly characterized by overactivity of a signal transduction pathway is neurofibromatosis. See, in general, The Merck Manual of Diagnosis and Therapy, 16th Edition, 1992. Modulation of Trk activity may also be useful in treating cancers of the nerve tissue, such as neuroblastoma, even if an abnormality is not found the signalling pathway.

Mutations in the Trk gene are also involved in cancer. Mutant Trk genes have been isolated from both colon and thyroid carcinomas. In general, the mutations involve a molecular rearrangement such that the transmembrane and cytoplasmic portions of the Trk gene remain intact, however, the normal extracellular ligand binding sequences have been replaced by foreign sequences such as those coding for tropomyosin. Martin-Zanca, D., *Nature* 319:743-748, 1986, Pulciani, S., *Nature* 300:539-542, 1982, Bongarzone, I., *Oncogene* 4:1457-1462, 1989 and Park, M., *Cell* 45:895-904, 1986. Such mutations may lead to abnormal signal transduction activity by stimulating activity even in the absence of the normal ligand and/or stimulating activity in an inappropriate (non-neuronal) cell type.

Several companies are attempting to treat neurodegenerative diseases such as amyotrophic lateral sclerosis by giving patients exogenous recombinant neurotrophic factors, such as BDNF, in the hope that the factor will stimulate new bgrowth and differentiation of nerve cells. The importance of the Trk gene family for the growth, differentiation and survival of nerve cells was recently demonstrated by experiments in mice. Gene targeting was used to generate mice with null mutations

in genes encoding each of the known Trk receptors, and in one case the gene encoding a Trk ligand, meaning that these mice would never express the target gene. Smeyne, R.J., et al., Nature 368:246-249, 1994, and Klein, R., et al., Nature 268:249-251, 1994. The mice all had severe neurological dysfunction and, in all cases, important types of neural tissue were absent.

The development and maintenance of cellular communication networks within the central and peripheral nervous system is regulated by neurotrophic factors, which through activation of specific cell surface receptors generate differentiation and survival signals in neuronal cell types. Binding of the neurotrophic factor to its receptor initiates a cellular signal transduction cascade involving diverse cytoplasmic components which eventually results in a specific nuclear response. Specific cellular responses in nerve cells can include, for example, neurite outgrowth, acquisition of Na+-based action potential and cell survival in serum-free medium.

The complex processes of cell growth, differentiation and cell survival are mediated by diverse and divergent signal transduction pathways. The multiple phosphorylated tyrosines found in activated RTKs serve as binding sites for different signaling components, which in turn modulate the transduction of a signal along a particular pathway. In the case of the NGF receptor Trk, it has been shown that specific phosphorylated tyrosines within the cytoplasmic portion of the receptor can bind to the signaling components phospholipase C-gamma (PLC-gamma), SHC and the non-catalytic subunit of phosphatidylinositol-3'-kinase, p85. Obermeier, A., *EMBO Journal* 12:933-941, 1993 and Obermeier, A., *J. Biol. Chem.* 268:22963-22966, 1993. These proteins in turn each stimulate different and distinct further downstream signaling components until instructions are finally transmitted to the cell

nucleus. For example, SHC has been shown to bind to the Grb2/SOS complex, which in turn allows the activation of *ras*. Activation of PLC-gamma, on the other hand, leads to the generation of phosphatidylinositol metabolites, such as inositol 1,4,5-triphosphate, which cause the release of calcium ions from intracellular compartments and the generation of diacyglycerol, the natural activator of PKC. See Fig. 2.

Dynamin and Signal Transduction

Measurement of the level of interaction of a DYN domain with a SH3 domain has been determined to be a useful measure of the existence of certain diseases or conditions. Such diseases or conditions are thus characterized by inappropriate interaction of a DYN domain with a SH3 domain. Diseases or conditions characterized by abnormal levels of interaction between a DYN domain and a SH3 domain can be alleviated to some extent by disrupting or promoting that interaction in vivo. In addition, useful agents for treatment of such diseases can be identified by standard screening protocols in which measurement of such interaction is determined. For example, such an agent may be a peptide which either comprises, consists of, or consists essentially of the DYN domain.

We have identified proteins of 110, 80, 65, and 43 kD in human embryonic fibroblasts that bind specifically to the SH3 domain of phospholipase Cγ (PLCγ), a primary substrate of receptor tyrosine kinases, and characterized the 110 kD band as the microtubule-activated GTPase dynamin. In addition, dynamin binds the son of sevenless adaptor protein GRB-2 with even higher affinity. This interaction does not require the dynamin GTPase function and involves a proline-rich target sequence between residues 812-820 of dynamin.

We used a fusion protein between the SH2-SH2-SH3 portion of PLCγ and glutathione-S-transferase bound to glutathione Sepharose as an affinity matrix for the

purification of SH3 binding proteins from human 293 fibroblasts. This resulted in the identification of four bands of 43 kD, 65 kD, 80 kD, and 110 kD, of which the 110 kD protein appeared to have the highest affinity. In vitro binding to PLCγ-SH3, PLCγ-SH2-SH2, and PLCγ-SH2-SH2-SH3 fusion proteins revealed that p110 specifically interacts with the PLCγ SH3 domain. Upon affinity purification using the GST-PLCγ-SH3 fusion protein and partial amino acid sequence analysis, this protein was identified as the microtubule-activated GTPase dynamin.

Dynamin was identified and purified with GST-GRB-2 fusion proteins. Here, efficient binding requires a GST fusion with both the GRB-2 SH2 domain and the N-terminal SH3 domain. Comparison of dynamin interactions with SH2/SH3-containing signal-transducing proteins revealed a hierarchy, with GRB-2 and PLCγ exhibiting similar affinities in intact transfected cells and GAP, p85/PI3'-K, and SHC showing no binding above the background of the control. In vitro, however, using GST-fusion proteins with SH3 domain-containing protein fragments, binding of dynamin to p85/PI3'-K was detectable at low levels, yet both GRB-2 and PLCγ SH3 domain also displayed higher affinities under these conditions.

Dynamin is a member of a GTP-binding protein family that includes Mx-1-related proteins, which are interferon-inducible and confer resistance to virus infection, MGM1, which plays a role in yeast mitochondrial DNA maintenance, and VPS1P, a protein involved in vacuolar protein sorting in yeast. Vallee, *J. Muscle Research and Cell Motility* 13:493-496, 1992. Mutations within the GTP binding site of dynamin were found to block receptor-mediated endocytosis of transferrin at a stage following coat assembly and preceding the sequestration of ligands into deeply invaginated coated pits. van der Bliek et al., *J. Cell Biol.* 122:553-563, 1993; Herskovits et al., *J. Cell Biol.* 122:565-578, 1993.

Consequently, our demonstration of dynamin interaction with PLCγ and GRB-2 indicates an involvement of these factors in RTK internalization. Upon ligand activation, PLCγ and GRB-2, as well as other proteins, transiently form complexes with certain RTKs, followed by rapid internalization and degradation or recycling of the receptor to the cell surface. Carpenter et al., *Annu. Rev. Biochem.* 48:193-216, 1979; Marshall, *J. Biol. Chem.* 260:4136-4144, 1985; Ullrich et al., *Cell* 61:203-212, 1990. While it has been shown that the receptor tyrosine kinase function is required for internalization, the molecular details of this process have not been understood. Honegger et al., *Cell* 51:199-209, 1987; Glenney et al., *Cell* 52:675-684, 1988.

Our findings indicate that ligand-dependent binding of PLCγ connects the receptor to the cellular internalization and degradation machinery involving dynamin. This conclusion is supported by previous studies which identified a so called Caln domain between residues 973 and 999 within the C-tail region of the human EGF-R as being required for EGF-dependent internalization, downregulation, and degradation. Chen et al., *Cell* 59:33-43, 1989. This region includes tyrosine 992, the high affinity binding site for PLCγ (Rotin et al., *EMBO J.* 11:559-567, 1992), indicating that EGF-Rs that are disabled in their interaction with PLCγ cannot couple to dynamin and are therefore not, or inefficiently, internalized. Alternatively, uncoupling of PLCγ from EGF receptors prevents the formation of second messengers that activate protein kinase C (PKC) (reviewed in Hug et al., *Biochem. J.* 291:329-343, 1993). Moreover, PKC may modulate endocytosis through the phosphorylation and dephosphorylation of dynamin, which is known to regulate dynamin GTPase activity in synapses. (van der Bliek et al., *J. Cell Biol.* 122:553-563, 1993; Robinson et al., *Nature* 365:163-166, 1993)

The present results indicate that SH3 domains

play a role in targeting signaling molecules to specific subcellular locations. Purified dynamin was found to exhibit GTPase activity, which was stimulated by microtubules in vitro and led to the formation of microtubular bundles in a nucleotide-dependent manner. Vallee, *J. Muscle Research and Cell Motility* 13:493-496, 1992. This observation provides the basis for a model in which dynamin mediated the movement of molecules along a network of microtubules. Without being bound by a particular understanding as to the operation of the present invention, it is presently believed that dynamin links signal transduction components to the cytoskeleton and mediates their transport to specific subcellular locations. This conclusion is supported by Bar Sagi et al. (*Cell* 74:83-91, 1993), who demonstrated that the SH3 domain of PLCγ is responsible for targeting to the cytoskeletal actin network, while the SH3 domains of GRB-2 guides this molecule to membrane ruffles.

In the context of PLCγ and GRB-2 interactions with dynamin, these observations indicate actin as an alternative to the involvement of microtubules in playing a role in the transport of signalling factors to subcellular locations. Such a scenario sheds new light on the role of PLCγ and that of the products of its catalytic activity, IP3 and DAG, as well as their targets, the [Ca^{2+}] regulator IP3 receptor and protein kinase C, respectively, in this process. Dynamin may also participate in receptor stimulus-mediated translocation of PLCγ and the GRB-2/SOS complex from cytoplasmic locations to the phosphorylated receptor to allow hydrolysis of membrane-associated PIP_2 and coupling to ras, respectively.

Competition experiments using synthetic peptides corresponding to each of the four proline-rich sequences in dynamin clearly revealed that peptide 3 (APPVPSRPG) (SEQ. I.D. NO. 4) is most potent in inhibiting the interaction with the SH3 domain of PLCγ. The

SOS interaction site with the SH3 domains of GRB-2 is (PPVPPR (SEQ. I.D. NO. 5) (Egan et al. *Nature* 363:45-51, 1993; Li et al., *Nature* 363:85-8812, 1993), suggesting that PLCγ and GRB-2 interact with dynamin via the same site. This is supported by the observation that GRB-2 SH3 mutants that are impaired in their interaction with SOS also fail to coimmunoprecipitate dynamin and that a GST-dynamin fusion protein containing the proline-rich APPVPSR (SEQ. I.D. NO. 6) motif clearly mediated binding of GRB-2 *in vitro*. Less pronounced yet detectable was the inhibitory effect of peptides 1 (APAVPPARPG) (SEQ. I.D. NO. 7) and 4 (PFGPPPQVPS) (SEQ. I.D. NO. 8) on the PLCγ-SH3/dynamin interaction, the former of which is contained in a sequence that was proposed recently by Gout et al. (*Cell* 75:25-36, 1993) to represent a PLCγ and GRB-2 binding site of dynamin. The APPVPSRPG sequence (SEQ. I.D. NO. 9), which was omitted in the Gout *et al.* study, contains the major SH3 binding site for PLCγ and GRB-2, and the APAVPPARPG (SEQ. I.D. NO. 10) and PFGPPPQVPS (SEQ. I.D. NO. 11) peptides contain binding motifs for other SH3 proteins such as p85 or as yet unidentified signal transducers. The promiscuity of SH3-target interactions suggested by these observations parallels that of SH2 domains that bind with distinct affinities to different RTKs. Taken together, the diversity of SH2 and SH3 domain-mediated protein-protein interactions may represent the biochemical basis for the definition of RTK- and cell type-specific signals.

MP Domains and Signal Transduction

The present invention is based in part upon the discovery of novel interactions between SH3 domains and MP domains, specifically the interaction between heterogenous ribonucleoprotein MP domains and SH3 domains and the interaction between vav protein SH3 domains and MP domains.

The role of the proto-oncogene product $p95^{vav}$ in

signal transduction was characterized by its interactions with proteins that may represent components of a novel signaling pathway. We demonstrate here stable association of $p95^{vav}$ with the heterogeneous ribonucleoprotein K (hnRNP-K), a protein that not only was found to be part of hnRNP particles, but has also been implicated in transcriptional regulation of the *c-myc* gene. Through the PLPPPPPPRG sequence (SEQ. I.D. NO. 2), hnRNP-K specifically interacts with the SH3 domain of $p95^{vav}$ and thus represents a novel SH3-binding protein that may be capable of translating cell surface receptor signals through $p95^{vav}$ activation into regulatory events on the level of gene expression.

The RNA binding protein hnRNP-K was identified as a vav SH3 domain interaction target. In light of the roles of hnRNP-K in mRNA transport and myc gene regulation, our findings present a novel signaling pathway that connects *vav*/receptor engagement at the plasma membrane with regulatory events taking place in the cytoplasm and the nucleus.

While vav is exclusively expressed in hematopoietic cells (Katzav et al. *EMBO J.* 8:2283-2290, 1989), hnRNP-K is ubiquitously expressed in both hematopoietic cells and non-hematopoietic cells (Matunis et al., *Mol. Cell. Biol.* 12:164-171, 1992). Thus, for non-hematopoietic cells, one may postulate the existence of a vav homologue that analogously utilizes hnRNP-K for the transmission of cellular signals. The concept that a component of hnRNP particles may also take part in signal transduction processes rather than fulfilling only "housekeeping" functions was recently supported by the demonstration of transcription factor activity of hnRNP-K on the *c-myc* gene CT-promoter element (Takimoto et al., *J. Biol. Chem.* 268:18249-18258, 1993). Our findings indicate that vav, through SH3-mediated association with hnRNP-K, utilizes a direct pathway to signal to the nucleus. While the more N-terminal SH3 domain of vav, which is not able

to bind hnRNP-K, might be responsible for directing vav to specific subcellular sites, as has been shown for the SH3 domains of GRB-2 and PLCγ (Bar-Sagi et al., *Cell* 79:83-91, 1993), the C-terminal SH3 domain might function as a signal transfer module, connecting cell surface receptor-triggered events to the hnRNP-K and on into the nucleus.

While in accordance with its action as a GRF for ras, $p95^{vav}$ is predominantly found in the cytoplasm, both immunofluorescence (Margolis et al., *Nature* 356:71-74, 1992) and subcellular fractionation demonstrate additional localization in the nucleus, where it may participate in transcriptional regulation of primary response genes as part of a complex with hnRNP-K. Conversely, significant amounts of hnRNP-K are also detectable in the cytoplasm, where it likely plays a role in mRNA transport and possibly translation. The vav/hnRNP-K interaction may take place in the cytoplasm or the nucleus and *vav* may also participate in modulation of hnRNP-K functions other than gene regulation. Further support for the involvement of RNA binding proteins such as hnRNP-K in receptor-induced signaling pathways have been recently provided by Fumagalli *et al.* (Fumagalli et al., *Nature* 368:871-874, 1994, not admitted to be prior art) and Taylor and Shalloway (Taylor and Shalloway, *Nature* 368:867-871, 1994, not admitted to be prior art), who described the interaction of p68 with src and implicated this association in mitotic regulation.

Direct mediation of signaling functions by SH3 domains have previously been reported for GAP. As shown in *Xenopus* oocytes (Duchesne et al., *Science* 259:525-528, 1993) and mouse fibroblasts (Medema et al., *Mol. Cell. Biol.* 12:3425-3430, 1992), the GAP SH2 and SH3 domains are essential for delivering a ras-dependent signal to the nucleus, which in fibroblasts leads to *fos* gene induction (Medema et al., *Mol. Cell. Biol.* 12:3425-3430, 1992). The SH2 domain of GAP interacts with p62, a protein that has recently been shown to have the characteristics of an RNP

(Wong et al., *Cell* 69:551, 1992).

Both p62 and hnRNP-K, contain the so called KH-domain, which was found in several RNA binding proteins (Siomi et al., *Nucl. Acids Res.* 21:1993-1198, 1993, Gibson et al., *FEBS* 324:361-366, 1993) and which is shared by the fragile X gene product (Siomi et al., *Cell* 74:291-298, 1993). Our findings strongly suggest a novel mode of signal transmission connecting primary RTK substrates vav and GAP with RNA binding proteins hnRNP-K and p62, and nuclear gene regulation mechanisms involving the protooncogenes *myc* and *fos*, respectively.Compositions

The present invention relates to removing or reducing an abnormality in a signal transduction pathway, wherein the signal transduction pathway contains SHC and Trk. The present invention also relates to compositions and methods for the treatment of disorders which involve modulating the activity and/or level of individual components, and relates to methods for the identification of agents for such treatments. Additionally, the present invention relates to methods and compositions for prognostic evaluation of such disorders.

Ligand/Receptor Complexes

Described herein are compositions and methods for the prevention, prognostic evaluation, and treatment of disorders in which the individual moleculaes listed above may be involved. Also described are compositions and methods for the prevention, prognostic evaluation and treatment of such disorders.

First, methods and compositions for the treatment of such disorders are described. Such methods and compositions may include, but are not limited to the agents capable of decreasing or inhibiting the interaction between the individual molecules and agents capable of inhibiting or decreasing the activity of such complexes, agents capable of modulating the activity and/or level of individual components of the proteins, and the use and administration of such agents. Agents capable of modu-

lating the activity and/or level of interation include those agents that inhibit or decrease the dephosphorylating activity of tyrosine phosphatases.

Second, methods are described for the identification of such agents. These methods may include, for example, assays to identify agents capable of disrupting or inhibiting or promoting the interaction between components of the complexes, and may also include paradigms and strategies for the rational design of drugs capable of disruption and/or inhibition and/or promotion of such complexes.

The complexes involved in the invention include signalling proteins such as SHC and a member of the Trk family of RTKs or derivatives thereof, complexes between particular SH3 and MP domains, and complexes between SH3 and DYN domains, as described below. Under standard physiological conditions, the components of such complexes are capable of forming stable, non-covalent attachments with one or more of the other complex components. Methods for the purification and production of such protein complexes, and of cells that exhibit such complexes are described below.

The complexes involved in the invention also include tyrosine phosphatases responsible for dephosphorylating activated receptors, thus modulating the abiliyty to bind to other signal transduction components. Identification of such tyrosine phosphatase(s) may be accomplished using techniques known to one skilled in the art. For example, cells that do not normally express Trk can be co-transfected with a Trk gene and a gene encoding a tyrosine phosphatase. After exposure to a Trk ligand, such as NGF, the cells are lysed, then the Trk receptor is isolated, for example by immunoprecipitation, and reacted with an anti-phosphotyrosine antibody. No phosphotyrosine will be detected on Trk receptors co-expressed with a tyrosine phosphatase capable of dephophorylating activated Trk.

Disruption of Protein Complexes

Disruption of complexes, for example by decreasing or inhibiting or promoting the interactions between component members of such a complex may have differing modulatory effects on the event involved, depending on the individual protein complex. "Disruption", as used here, is meant to refer not only to a physical separation of protein complex components, but also refers to a perturbation of the activity of the complexes, regardless of whether or not such complexes remain able, physically, to form. "Activity", as used here, refers to the function of the protein complex in the signal transduction cascade of the cell in which such a complex is formed, i.e., refers to the function of the complex in effecting or inhibiting a transduction of an extracellular signal into a cell. For example, the effect of complex disruption may augment, reduce, or block a signal normally transduced into the cell. Likewise, depending on the disorder involved, either augmentation, reduction, or blockage of a signal normally transduced into the cell will be desirable for the treatment of the disorder.

A disorder involving a complex may, for example, develop because the presence of such a complex brings about the aberrant inhibition of a normal signal transduction event. In such a case, the disruption of the complex would allow the restoration of the usual signal transduction event. Further, an aberrant complex may bring about an altered subcellular adapter protein localization, which may result in, for example, dysfunctional cellular events. An inhibition of the complex in this case would allow for restoration or maintenance of a normal cellular architecture. Still further, an agent or agents that cause(s) disruption of the complex may bring about the disruption of the interactions among other potential components of a complex.

Nucleotide sequences encoding peptide agents

which are to be utilized intracellularly may be expressed in the cells of interest, using techniques which are well known to those of ordinary skill in the art. For example, expression vectors derived from viruses such as retroviruses, vaccinia virus, adenoviruses, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery and expression of such nucleotide sequences into the targeted cell population. Methods for the construction of such vectors are well known. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Complex-binding domains can be identified using, for example, techniques such as those described in Rotin et al. (Rotin, D. et al., EMBO J. 11:559-567), Songyang et al. (Songyang, S. et al., 1993, Cell 72:767-778), Felder, S. et al., 1993, Mol. Cell. Biol. 13:1449-1455, Fantl, W.J. et al., 1992, Cell 69:413-422, and Domchek, S.M. et al., 1992, Biochemistry 31:9865-9870.

Alternatively, antibodies capable of interfering with complex formation may be produced as described below and administered for the treatment of disorders involving a component capable of forming a complex with another protein. For example, neutralizing antibodies which are capable of interfering with ligand binding may be administered using standard techniques. Alternatively, nucleotide sequences encoding single-chain antibodies may be expressed within the target cell population by utilizing, for example, techniques such as those described in Marasco et al. (Marasco, W. et al., 1993, Proc. Natl. Acad. Sci. USA 90:7889-7893).

Agents which act intracellularly to interfere with the formation and/or activity of the protein complexes of the invention may also be small organic or inorganic compounds. A method for identifying these and other

intracellular agents is described below.

Antibodies to Complexes

Described herein are methods for the production of antibodies which are capable of specifically recognizing a complex or an epitope thereof, or of specifically recognizing an epitope on either of the components of the complex, especially those epitopes which would not be recognized by the antibody when the component is present separate and apart from the complex. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a complex in a biological sample, or, alternatively, as a method for the inhibition of a complex formation, thus, inhibiting the development of a disorder.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as a complex, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, various host animals may be immunized by injection with the complex including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

A monoclonal antibody, which is a substantially homogeneous population of antibodies to a particular antigen, may be obtained by any technique which provides for

the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983 Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,-778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce complex-specific single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragment of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which contain specific binding

sites of a complex may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to the PTK/adapter complex.

One or more components of a protein complex may be present at a higher than normal cellular level (i.e., higher than the concentration known to usually be present in the cell type exhibiting the protein complex of interest) and/or may exhibit an abnormally increased level of cellular activity (i.e., greater than the activity known to usually be present in the cell type exhibiting the protein complex of interest).

For example, the gene encoding a protein complex component may begin to be overexpressed, or may be amplified (i.e., its gene copy number may be increased) in certain cells, leading to an increased number of component molecules within these cells. Additionally, a gene encoding a protein complex component may begin to express a modified protein product that exhibits a greater than normal level of activity. "Activity", here, refers to the normal cellular function of the component, either enzymatic or structural whose function may include, for example, bringing two or more cellular molecules into the appropriate proximity.

Such an increase in the cellular level and/or activity of a protein complex may lead to the development of a disorder. Treatment of such disorders may, therefore, be effectuated by the administration of agents which decrease the cellular level and/or the activity of the overexpressed and/or overactive protein complex component.

Techniques for decreasing the cellular level and/

or the activity of one or more of the protein complex components of interest may include, but are not limited to antisense or ribozyme approaches, and/or gene therapy approaches, each of which is well known to those of skill in the art.

Antisense and Ribozyme Approaches

Included in the scope of the invention are oligoribonucleotides, including antisense RNA and DNA molecules and ribozymes that function to inhibit translation of one or more components of a protein complex. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the relevant nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead or other motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding protein complex components.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease

protection assays. See, Draper PCT WO 93/23569.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. See, Draper, id. hereby incorporated by reference herein. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5′ and/or 3′ ends of the molecule or the use of phosphorothioate or 2′ O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Gene Therapy

Target cell populations may be modified by introducing altered forms of one or more components of the protein complexes in order to modulate the activity of such complexes. For example, by reducing or inhibiting a complex component activity within target cells, an abnormal signal transduction event(s) leading to a condition may be decreased, inhibited, or reversed. Deletion or missense mutants of a component, that retain the ability to interact with other components of the protein complexes but cannot function in signal transduction may be used to inhibit an abnormal, deleterious signal transduction

event.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of nucleotide sequences encoding recombinant protein complex components into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked DNA or in reconstituted system e.g., liposomes or other lipid systems for delivery to target cells (See e.g., Felgner et al., 1989, Nature 337:387-8).

Pharmaceutical Formulations and Modes of Administration

The particular compound, antibody, antisense or ribozyme molecule that affects the protein complexes and the disorder of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

In treating a patient exhibiting an oncogenic disorder of interest, a therapeutically effective amount of a agent or agents such as these is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and

it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the oncogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic

evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," 1990, 18th ed., Mack Publishing Co., Easton, PA. Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries,

suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active

compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used

orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Identification of Agents

The complexes, components of such complexes, functional equivalents thereof, and/or cell lines that express such components and exhibit such protein complexes may be used to screen for additional compounds, antibodies, or other molecules capable of modulating the signal transduction event such complexes are involved in. Methods for purifying and/or producing such complexes, components of the complexes, functional equivalents thereof, and/or cell lines are described herein. The compounds, antibodies, or other molecules identified may, for example, act to disrupt the protein complexes of the invention (i.e., decrease or inhibit interactions between component members of the complexes, thereby causing physical separation of the components, and/or perturbing the activity of the complexes) or may lower the cellular level and/or decrease the activity of one or more of the components of such complexes.

Such compounds may include, but are not limited to, peptides made of D- and/or L-configuration amino acids (in, for example, the form of random peptide libraries; see Lam, K.S. et al., 1991, Nature 354:82-84), phosphopeptides (in, for example, the form of random or partially degenerate, directed phosphopeptide libraries, see Songyang, Z. et al., 1993, Cell 767-778), antibodies, and small organic or inorganic molecules. Synthetic compounds, natural products, and other sources of potential-

ly biologically active materials may be screened in a variety of ways, as described herein. The compounds, antibodies, or other molecules identified may be used as oncogenic disorder treatments, as described herein.

Compounds that bind to individual components, or functional portions of the individual components of the complexes (and may additionally be capable of disrupting complex formation) may be identified.

One such method included within the scope of the invention is a method for identifying an agent to be tested for an ability to modulate a signal transduction pathway disorder. The method involves exposing at least one agent to a protein comprising a functional portion of a member of the protein complex for a time sufficient to allow binding of the agent to the functional portion of the member; removing non-bound agents; and determining the presence of the compound bound to the functional portion of the member of the protein complex, thereby identifying an agent to be tested for an ability to modulate a disorder involving a polypeptide complex.

The term "protein" refers to a compound formed of 5-50 or more amino acids joined together by peptide bonds. An "amino acid" is a subunit that is polymerized to form proteins and there are twenty amino acids that are universally found in proteins. The general formula for an amino acid is H_2N-CHR-COOH, in which the R group can be anything from a hydrogen atom (as in the amino acid glycine) to a complex ring (as in the amino acid tryptophan).

A functional portion of an individual component of the complexes may be defined here as a protein portion of an individual component of a complex still capable of forming a stable complex with another member of the complex under standard cellular and physiological conditions. For example, a functional portion of a component may include, but is not limited to, a protein portion of the RTK of the Trk family which is still capable of stably

binding a corresponding signalling component domain of an associated protein, and thus is still capable of forming a complex with that protein. Further, in the case of the catalytic domains of the individual components of the invention, a functional portion of a catalytic domain may refer to a protein still capable of stably binding a substrate molecule under standard physiological conditions.

One method utilizing this approach that may be pursued in the isolation of such complex component-binding molecules would include the attachment of a component molecule, or a functional portion thereof, to a solid matrix, such as agarose or plastic beads, microtiter wells, petri dishes, or membranes composed of, for example, nylon or nitrocellulose, and the subsequent incubation of the attached component molecule in the presence of a potential component-binding compound or compounds. Attachment to said solid support may be direct or by means of a component specific antibody bound directly to the solid support. After incubation, unbound compounds are washed away, component-bound compounds are recovered. By utilizing this procedure, large numbers of types of molecules may be simultaneously screened for complex component-binding activity.

The complex components which may be utilized in the above screening method may include, but are not limited to, molecules or functional portions thereof, such as catalytic domains, phosphorylation domains, extracellular domains, or portions of extracellular domains, such as ligand-binding domains, and adaptor proteins, or functional portions thereof. The peptides used may be phosphorylated, e.g., may contain at least one phosphorylated amino acid residue, preferably a phosphorylated Tyr amino acid residue, or may be unphosphorylated. A phosphorylation domain may be defined as a peptide region that is specifically phosphorylated at certain amino acid residues. A functional portion of such a phosphorylation domain may be

defined as a peptide capable of being specifically phosphorylated at certain amino acids by a specific protein.

Molecules exhibiting binding activity may be further screened for an ability to disrupt protein complexes. Alternatively, molecules may be directly screened for an ability to promote the complexes. For example, in vitro complex formation may be assayed by, first, immobilizing one component, or a functional portion thereof, of the complex of interest to a solid support. Second, the immobilized complex component may be exposed to a compound such as one identified as above, and to the second component, or a functional portion thereof, of the complex of interest. Third, it may be determined whether or not the second component is still capable of forming a complex with the immobilized component in the presence of the compound. In addition, one could look for an increase in binding.

Additionally, complex formation in a whole cell may be assayed by utilizing co-immunoprecipitation techniques well known to those of skill in the art. Briefly, a cell line capable of forming a complex of interest may be exposed to a compound such as one identified as above, and a cell lysate may be prepared from this exposed cell line. An antibody raised against one of the components of the complex of interest may be added to the cell lysate, and subjected to standard immunoprecipitation techniques. In cases where a complex is still formed, the immunoprecipitation will precipitate the complex, whereas in cases where the complex has been disrupted, only the complex component to which the antibody is raised will be precipitated.

A preferred method for assessing modulation of complex formation within a cell utilizes a method similar to that described above. Briefly, a cell line capable of forming a complex of interest is exposed to a test compound. The cells are lysed and the lysate contacted with an antibody specific to one component of the complex, said

antibody having been previously bound to a solid support. Unbound material is washed away, and the bound material is exposed to a second antibody, said second antibody binding specifically to a second component of the complex. The amount of second antibody bound is easily detected by techniques well known in the art. Cells exposed to an inhibitory test compound will have formed a lesser amount of complex compared to cells not exposed to the test compound, as measured by the amount of second antibody bound. Cells exposed to a test compound that promotes complex formation will have an increased amount of second antibody bound.

The effect of an agent on the differentiation capability of the complex of interest may be directly assayed. Such agents may, but are not required to, include those agents identified by utilizing the above screening technique. For example, an agent or agents may be administered to a cell such as a neuronal cell, capable of forming a complex, for example, which, in the absence of any agent, would not lead to the cell's differentiation. The differentiation state of the cell may then be measured either in vitro or in vivo. One method of measurment may involve observing the amount of neurile growth present.

Agents capable of disrupting complex formation and capable of reducing or inhibiting disorders, which involve the formation of such complexes, or which involve the lack of formation of such complexes, may be used in the treatment of patients exhibiting or at risk for such disorders. A sufficient amount of agent or agents such as those described above may be administered to a patient so that the symptoms of the disease or condition are reduced or eliminated.

Purification and Production of Complexes

Described in this Section are methods for the synthesis or recombinant expression of components, or fragments thereof, of the protein complexes of the inven-

tion. Also described herein are methods by which cells exhibiting the protein complexes of the invention may be engineered.

Purification Methods

The complexes of the invention may be substantially purified, i.e., may be purified away from at least 90% (on a weight basis), and from at least 99%, if desired, of other proteins, glycoproteins, and other macromolecules with which it is associated. Such purification can be achieved by utilizing a variety of procedures well known to those of skill in the art, such as subjecting cells, tissue or fluid containing the complex to a combination of standard methods, for example, ammonium sulfate precipitation, molecular sieve chromatography, and/or ion exchange chromatography.

Alternatively, or additionally, a complex may be purified by immunoaffinity chromatography using an immunoadsorbent column to which an antibody is immobilized which is capable of binding to one or more components of the complex. Such an antibody may be monoclonal or polyclonal in origin. Other useful types of affinity purification for the protein complex may utilize, for example, a solid-phase substrate which binds the catalytic kinase domain of a protein, or an immobilized binding site for noncatalytic domains of the components of the complex, which bind in such a manner as to not disrupt the complex. The complex of the present invention may be biochemically purified from a variety of cell or tissue sources.

Synthesis and Expression Methods

Methods for the synthesis of polypeptides or fragments thereof, which are capable of acting as components of the complexes of the present invention, are well-known to those of ordinary skill in the art. See, for example, Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., NY, which is incorporated herein, by reference, in its entirety.

Components of a complex which have been sepa-

rately synthesized or recombinantly produced, may be reconstituted to form a complex by standard biochemical techniques well known to those skilled in the art. For example, samples containing the components of the complex may be combined in a solution buffered with greater than about 150mM NaCl, at a physiological pH in the range of 7, at room temperature. For example, a buffer comprising 20mM Tris-HCl, pH 7.4, 137mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate and 2mM EDTA could be used.

Methods for preparing the components of complexes of the invention by expressing nucleic acid encoding proteins are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. DNA and RNA synthesis may, additionally, be performed using an automated synthesizers. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the coding sequences of the components of the complexes of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the protein complexes of the invention. These include but are not limited to microorganisms such as bacteria (e.g., E.coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing protein coding sequences; yeast (e.g., Saccha-

romyces and Pichia) transformed with recombinant yeast expression vectors containing the protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the protein coding sequences coding sequence; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the complex being expressed. For example, when large quantities of complex proteins are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the protein coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa

protease cleavage sites so that the cloned protein can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The complex coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the PTK/adaptor complex coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the complex coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by <u>in vitro</u> or <u>in vivo</u> recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing proteins in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences.

In cases where an entire protein gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no

additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably coexpress both the proteins may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the protein encoding DNA independently or coordinately controlled by appropriate expression control elements (e.g., promoter, enhancer,

sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker.

Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which coexpress both the PTK and adaptor protein. Such engineered cell lines are particularly useful in screening and evaluation of compounds that affect signals mediated by the complexes.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

New members of the protein families capable of forming the complexes of the invention may be identified and isolated by molecular biological techniques well known in the art. For example, a previously unknown protein encoding gene may be isolated by performing a polymerase chain reaction (PCR) using two degenerate oligonucleotide

primer pools designed on the basis of highly conserved sequences within domains common to members of the protein family.

The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express complexes. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the sequences of a member of the PTK or adaptor subfamily. The PCR fragment may then be used to isolate a full length protein cDNA clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.). A general method for cloning previously unknown proteins has been described by Skolnik (Skolnik, E.Y., 1991, Cell 65:75) and Skolnik et al., (U.S. Patent Application Serial No. 07/643,237) which are incorporated herein, by reference, in their entirety, including drawings.

Derivatives of Complexes

Also provided herein are functional derivatives of a complex. By "functional derivative" is meant a "chemical derivative," "fragment," "variant," "chimera," or "hybrid" of the complex, which terms are defined below. A functional derivative retains at least a portion of the function of the protein, for example reactivity with an antibody specific for the complex, enzymatic activity or binding activity mediated through noncatalytic domains, which permits its utility in accordance with the present invention.

A "chemical derivative" of the complex contains additional chemical moieties not normally a part of the

protein. Covalent modifications of the protein complex or peptides are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues, as described below.

Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect or reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing primary amine containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group.

Furthermore, these reagents may react with the groups of lysine as well as the arginine alpha-amino group.

Tyrosyl residues are well-known targets of modification for introduction of spectral labels by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction carbodiimide (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residue are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful, for example, for cross-linking the component peptides of the complexes to each other or the complex to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl) dithiolpropioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016;

4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the Nterminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties may improve the stability, solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein complex and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990).

The term "fragment" is used to indicate a polypeptide derived from the amino acid sequence of the proteins, of the complexes having a length less than the full-length polypeptide from which it has been derived. Such a fragment may, for example, be produced by proteolytic cleavage of the full-length protein. Preferably, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the proteins to delete one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. Fragments of a protein, when present in a complex resembling the naturally occurring complex, are useful for screening for compounds that act to modulate signal transduction, as described below. It is understood that such fragments, when present in a complex may retain one or more characterizing portions of the native complex. Examples of such retained characteristics include: catalytic activity; substrate specificity; interaction with other molecules in the intact cell; regulatory

functions; or binding with an antibody specific for the native complex, or an epitope thereof.

Another functional derivative intended to be within the scope of the present invention is a complex comprising at least one "variant" polypeptide which either lack one or more amino acids or contain additional or substituted amino acids relative to the native polypeptide. The variant may be derived from a naturally occurring complex component by appropriately modifying the protein DNA coding sequence to add, remove, and/or to modify codons for one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such variants having added, substituted and/or additional amino acids retain one or more characterizing portions of the native complex, as described above.

A functional derivative of complexes comprising proteins with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary skill in the art. For example, the modified components of the functional derivatives may be produced using site-directed mutagenesis techniques (as exemplified by Adelman et al., 1983, DNA 2:183) wherein nucleotides in the DNA coding the sequence are modified such that a modified coding sequence is modified, and thereafter expressing this recombinant DNA in a prokaryotic or eukaryotic host cell, using techniques such as those described above. Alternatively, components of functional derivatives of complexes with amino acid deletions, insertions and/or substitutions may be conveniently prepared by direct chemical synthesis, using methods well-known in the art. The functional derivatives of the complexes typically exhibit the same qualitative biological activity as the native complexes.

Evaluation of Disorders

The protein complexes of the invention involved in disorders may be utilized in developing a prognostic

evaluation of the condition of a patient suspected of exhibiting such a disorder. For example, biological samples obtained from patients suspected of exhibiting a disorder involving a protein complex may be assayed for the presence of such complexes. If such a protein complex is normally present, and the development of the disorder is caused by an abnormal quantity of the complex, the assay should compare complex levels in the biological sample to the range expected in normal tissue of the same cell type.

Among the assays which may be undertaken may include, but are not limited to isolation of the protein complex of interest from the biological sample, or assaying for the presence of the complex y exposing the sample to an antibody specific for the complex, but non-reactive to any single, non-complexed component, and detecting whether antibody has specifically bound.

Alternatively, one or more of the components of the protein complex may be present in an abnormal level or in a modified form, relative to the level or form expected is normal, nononcogenic tissue of the same cell type. It is possible that overexpression of both components may indicate a particularly aggressive disorder. Thus, an assessment of the individual and levels of mRNA and protein in diseased tissue cells may provide valuable clues as to the course of action to be undertaken in treatment of such a disorder. Assays of this type are well known to those of skill in the art, and may include, but are not limited to, Northern blot analysis, RNAse protection assays, and PCR for determining mRNA levels. Assays determining protein levels are also well known to those of skill in the art, and may include, but are not limited to, Western blot analysis, immunoprecipitation, and ELISA analysis. Each of these techniques may also reveal potential differences in the form (e.g., the primary, secondary, or tertiary amino acid sequence, and/or post-translational modifications of the sequence)

of the component(s).

Examples

The examples below are non-limiting and are merely representative of various aspects and features of the present invention. The examples below demonstrate the interaction between SH3 domains and DYN domains both in vitro and in vivo using various methods, including coimmunoprecipitation and peptide competition techniques. The following examples also demonstrate the primary role played by the signaling component SHC, in the differentiation and survival of PC12 cells (ATCC CRL 1721). The examples below also demonstrate the interaction between SH3 domains and MP domains using various methods.

The role of PLCγ as a direct substrate of RTKs such as epidermal growth factor receptor (EGF-R) and platelet-derived growth factor receptor (PDGF-R) is well established. The significance of this RTK-PLCγ interaction and subsequent activation of its enzymatic function for the physiology of the cell, however, was unknown.

Recombinant human epidermal growth factor (EGF) was obtained from Intergen. The cDNAs for EP-R, the human EGF-R, PLCγ, GAP, p85, c-raf, SHC, GRB-2, GRB-2 1B (P49L), GRB-2 4B (G203R), dynamin, dynamin I, and dynamin III were cloned into a cytomegalovirus promoter-enhancer driven expression vector. The pGEX-SH2-SH2-SH3, pGEX-SH2-SH2, and pGEX-SH3 constructs have been described by Rotin *et al.* (*EMBO J.* 11:559-567, 1992).

PC12 cells represent a well established model system for NF-dependent neuronal differentiation. Tischler and Greene, *Nature* 258:341-342, 2975. In brief, a series of phosphotyrosine binding site mutants was made by substituting phenylalanine (F) for tyrosine (Y) at amino acid positions 785, 751, and 490 (PT-YF mutants), or all three positions (PT-Y3F), in the Trk cytoplasmic domain. These mutants were introduced into

PC12 cells to determine their biological effect.

Because of the absence of PDGF-receptors in PC12 cells (Heasley and Johnson, Molec. Biol. Cell 3:545-553, 1992), we chose an RTK chimera approach (Riedel et al., Nature, 324:68-70, 1986) to investigate individual elements of the NGF-R/Trk signal under PDGF control within its normal cell environment rather than in PC12-derived genetically altered mutant cell lines such as PC12-nnr5 (Green et al., J. Cell Biol. 102:830-843, 1986), which may be altered in their signal processing features. By introducing tyrosine to phenylalanine (YF) mutations either singly at positions 785, 751 and 490 or in all possible combinations into PT-R (figure 3), a chimeric receptor consisting of the βPDGF-R extracellular domain and the transmembrane and intracellular domains of Trk, we analyzed the significance of signalling pathways connected to SHC, PLCγ and p85/P13'-K phosphotyrosine target sites for NGF-R/Trk-specific signal transduction, which ultimately leads to neuronal differentiation and is essential for the maintenance of the neuronal network.

Materials and Methods

Construction of PT-receptors - An EcoR1/Mse1-restriction fragment containing the cDNA sequence for human βPDGF-receptor extracellular domain was fused to a cDNA sequence coding for a full-length rat Trk cDNA cloned into the Xba1 site of the pCMV polylinker. Obermeier, A., *J. Biol. Chem.* 268:22963-22966, 1993. For fusion, an Mse1 site was introduced by the 5'-PCR-primer sequence without changing the amino acid sequence. The chimeric cDNA was subcloned as a 3.3 kb EcoR1-fragment into pCMV-1 and into the retroviral vector pLEN. Adam, M.A., *J. Virol.* 65:4985-4990, 1991. Mutagenesis of PT-R cDNA was performed in the f1-origin containing pCMV-1 vector using a modified version of the method described by Kunkel,(Kunkel, N.E., *Proc. Natal. Acad. Sci. USA* 82:488-492, 1985 and Kunkel, N.E., *Methods Enzymol.*

154:367-382, 1987).employing M13K07 helper phages (Pharmacia). Oligonucleotides

5'-GGTATCACTGAAGAACTGTGGGTTCTC-3' (Y490F),

5'-GCGCATGATGGCGAAGACATCAGGAGG-3', (Y751F),

5'-CAGAACGTCCAGGAAACTCGTGGCGC-3' (Y785), and

5'-CTCCTTCAGTGCCTTGACAGCCACCAG-3' (KM)

were used to generate PT-YF mutants and PT-KM. After mutagenesis PT-cDNA fragments were subcloned via EcoR1 into pLEN.

Generation of PC12 transfectants and cell culture - Ecotrophic retroviruses were generated with the help of PA317 and GP+E-86 producer cell lines essentially as described previously. Redemann, N., *Molec. Cell. Biol.* 12:491-498, 1992. In case of pLEN-PT-Y785F and pLEN-PT-Y785/490F, ecotropic viruses were generated by transient transfection of 293 cells (ATCC CRL 1573) with pLEN constructs together with pSV-y-E-MLV helper virus DNA (Muller, A.J., *Molec. Cell. Biol.* 11:1785-1792, 1991) in a 5:3 ratio using the calcium phosphate coprecipitation technique (Chen, C. and Okayama H. *Molec. Cell. Biol.* 7:2745-2752, 1987); 48 hours after tranfection, virus containing supernatants were collected. Pure retrovirus-containing cell-free supernatants from G418-selected GP+E-86 or 293 cells were then added to PC12 cells 2x 4 hours in the presence of polybrene (4 ug/ml; Aldrich). Infected PC12 Cells were subsequently shifted from DMEM, 4500 g/l glucose (Gibco), 5% FCS (Gibco), 10% HS (horse serum, Boehringer Mannheim) to DMEM 2.5% FCS, 10% HS from platelet poor plasma (SIGMA), and selected for at least four weeks with G418. PC12 cells were grown at 37° C. 8% CO2 on collagen (type I, from rat tail; SIGMA) coated plastic dishes (Nunc).

PC-12 Differentiation Assays - PC-12 transfectants were seeded into collagen coated six-well dishes and after one day the medium was replaced by medium containing either 30 ng/ml PDGF-BB, 100 ng/ml NGF, or no ligand. Thereafter, medium and ligands were replaced

every two days.

Results

The effects of Trk cytoplasmic domain mutations were evaluated by visual examination of frequency and length of ligand-induced neurite outgrowth in direct comparison with PC-12/PT-R (chimera with no point mutations) and PC-12/Trk controls. As shown in Table 1, under identical conditions, PC-12 cells expressing one each of the various PT-YF mutants displayed clear differences in their neuronal differentiation responses after PDGF stimulation. The differentiation signal of the Trk cytoplasmic domain was severely impaired by mutation of the SHC binding site tyrosine 490, as shown by the phenotype of PC-12/PT-Y490F cells, which developed only few neurites of significantly reduced length when treated with PDGF. This experiment clearly shows that the interaction between activated Trk and SHC is essential for neuronal differentiation and survival.

Receptor Overexpressed	Binding Capacity			Differentiation response to:	
	PLCγ	P13'-K	SHC	PDGF	NGF
PT-R	+	+	+	++++	++++
PT-KM	-	-	-	-	++++
PT-Y490F	+	+	-	+	++++
PT-Y751F	+	-	+	++++	++++
PT-Y785F	-	+	-	++	++++
PT-Y785/490F	-	+	-	+/-	++++
PT-Y785/751F	-	-	+	+++	++++
PT-Y3F	-	-	-	+/-	++++
Trk	+	+	+	-	++++

Antibodies. For PLCγ, GAP, p85, c-raf, and dynamin, the 15 most C-terminal amino acids were used to raise polyclonal peptide antisera in rabbits. For SHC,

the 282 and for GRB-2 the 80 most C-terminal amino acids were expressed as pGEX fusion proteins in *E. coli* and after purification used as antigens in rabbits. EP-R and the human EGF-R were precipitated by the mouse monoclonal antibody 108.1. Honegger et al., *EMBO J.* 7:3053-3060, 1988.

Cell Culture. Human embryonic kidney fibroblasts (293; ATCC CRL 1573) were cultured in DMEM high glucose containing 10% fetal calf serum (FCS) and 2 mM L-glutamine (Gibco).

Transfection and Transient Expression. Transient transfections of 293 cells were performed essentially as described by Chen and Okayama (*Mol. Cell. Biol.* 7:2745-2752, 1987); Gorman *et al.* (*Virology* 171:377-385, 1989), and Aebersold et al. (*Proc. Natl. Acad. Sci. USA* 84:6970-697430, 1987). For metabolic labeling, the cells were washed twice with PBS and labeled overnight in methionine-free DMEM containing ^{35}S-methionine (1 mCi / 15 ml, Amersham), 0.5% FCS, and 2 mM L-glutamine.

Cell Lysis, Immunoprecipitation, and Gel Electrophoresis. Prior to lysis, the cells were treated with EGF (100 ng/ml) for 10 min. and washed once with PBS. Cells were lysed in 0.5 ml HNTG-buffer (Honegger et al., *EMBO J.* 7:3053-3060, 1988) containing 1% Triton X-100 and precleared by centrifugation at 12500 g for 10 min at 4°C.

For immunoprecipitations, the lysates were diluted with washing buffer (HNTG-buffer containing 0.1% Triton X-100) 1:2. 20 µl of protein-A-sepharose and 5 µl of the appropriate antibody were added and incubated for 4 h. at 4°C. Precipitates were washed four times with 1.5 ml washing buffer, each followed by the addition of SDS sample buffer and boiling for 5 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was then performed on 7, 5, 10, and 12.5% gels. For subsequent Western blot analysis, the separated proteins

were transferred to nitrocellulose (Schleicher and Schnell and incubated with the respective antiserum. Protein bands were made visible using horseradish peroxidase-coupled goat anti-mouse and goat anti-rabbit antibodies and the ECL™ (Amersham) detection method.

Purification and Sequence Analysis. About $5x10^{10}$ 293 cells were lysed in 100 ml HNTG buffer containing 1% Triton-X100. Lysates were incubated overnight with the glutathione-sepharose immobilized on the glutathione-S-transferase fusion protein GST-PLCγ-SH2-SH2-SH3. After washing with 10 vol. PBS, affinity-purified 293 cell proteins were eluted by RIPA buffer (50 mM Tris/Cl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% desoxycholate, 1% Triton-X100) and further analyzed.

Purification of the p110 GRB-2 binding protein was achieved by affinity chromatography using GRB-2-GST fusion protein covalently coupled to glutathione-agarose beads by the crosslinker dimethylimelidate. Lysate from 10^9 HER14 cells was precleared with GST crosslinked to glutathione-agarose for 1.5 h at 4°C and then incubated with GRB-2 beads (10 μg protein/ml lysate) for 2 h at 4°C. The beads were then washed 8x in RIPA buffer containing 200 μM orthovanadate. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, visualized by Ponceau S (Sigma) staining. Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after *in situ* protease digestion as previously described (Lee et al., *EMBO J.* 8:1369-1375, 1989) was obtained after blotting on nitrocellulose. Protein bands were excised from the membrane and digested with Lys-C protease (Boehringer) at 37°C for 20 h at a 1:20 enzyme to substrate ratio.

The products were then fractionated on an Applied Biosystems model 130 HPLC and RP300 reversed phase cartridge. Peptides were eluted using 0.1% trifluoric acid (buffer A and 0.08% trifluoroacetic acid

in acetonitrile/H_2O (70/30; buffer B) with a linear gradient of 5% to 95% buffer B. Peptides were subjected to automated Edman degradation using an Applied Biosystems model 477A protein sequencer with an on-line model 120A PTH analyzer.

The affinity-purified 110 kD protein was transferred to nitrocellulose membranes and digested *in situ* as described by Lee et al. (*EMBO J.* 8:1369-1375, 1989) using lys C protease (Boehringer Mannheim) at a molar enzyme to protein ratio of ~ 1:20. Resulting peptides were isolated using an RP-300 reversed phase cartridge on an Applied Biosystems Model 130A HPLC system. Peptides were subjected to automated Edman degradation using an Applied Biosystems Model 477A protein sequencer with on line PTH analyser.

Competition Experiments. Peptides 1, 2, 3, and 4 correspond to rat dynamin amino acids 785-794, 797-806, 812-820 and 825-834, respectively, and were synthesized by standard solid phase procedures. In order to enhance the solubility, each peptide was modified with arginine residues on both ends. Purified GST-PLCγ-SH3 fusion protein was bound to glutathionine-sepharose and washed 3 times with washing buffer (see cell lysis) containing 0.1% Triton X-100. Subsequently, metabolically labeled cell extracts containing overexpressed dynamin were added in the absence or presence of peptides at concentrations of 1 mM, 100 μM and 10 μM, respectively. After incubation for two hours at 4°C, the samples were washed four times with washing buffer and separated by SDS-PAGE, followed by exposure to film.

Cell lines and cell lysis.

Jurkat cells were maintained in RPMI medium supplemented with 2 mM glutamine, 10% FCS and antibiotics. Cells were lysed by shock-freezing and thawing in SolA-buffer, containing 10 mM Tris/HCl pH

7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 0.5 mM EDTA, 0.5 mM EGTA, and 1x Aprotinin. 293 human embryonic kidney fibroblasts were maintained in DMEM medium supplemented with 2 mM glutamine, 10% FCS and antibiotics. Lysis was performed as described (Lammers et al., *J. Biol. Chem.* 268:22456-22462, 1993).

Construction, expression, purification and biotinylation of GST fusion proteins.

GST fusion protein expression vectors were obtained by amplifying the respective domains of vav (as indicated in Fig. 1) by PCR and cloning into pGEX2T (Pharmacia). Expression, purification and biotinylation of GST fusion proteins were essentially done as previously described (Mayer et al., *Proc. Natl. Acad. Sci. USA* 88:627-631, 1991).

Far Western Blotting.

The procedure was performed essentially as previously described (Mayer et al., *Proc. Natl. Acad. Sci. USA* 88:627-631, 1991). Briefly, Jurkat cell lysates were separated by SDS-PAGE, blotted onto NC-membranes, blocked with TBST-0.2% gelatin and incubated overnight with biotinylated GST-fusion protein at a concentration of 1 mg/ml TBST-gelatin. Blots were developed with streptavidin-coupled alkaline phosphatase (Boehringer, Mannheim) and NBT plus X-P as substrates as recommended by the manufacturer.

Far Western blot analysis of Jurkat cell extracts with biotinylated GST-fusion proteins were obtained using GST protein alone as a control. Binding of p65 to a vavSH2/SH3 affinity matrix was determined. Total lysate (L), the supernatant over the matrix after binding of the lysate (S) and proteins eluting at increasing KCl concentrations were resolved by SDS-PAGE and assayed by Far Western Blotting with biotinylated vav SH2/SH3.

Affinity chromatography.

For GST-fusion protein affinity chromatography,

between 1 and 10 μg of purified GST-fusion protein and 40 to 100 μl of glutathione-sepharose 4B-suspension (Pharmacia) were added to cell lysates that had been diluted 1:1 with either TBST or HNTG buffer to keep the Triton X100 concentration low. After incubation for 3-12 h at 4°C, beads were washed extensively with either TBST or HNTG buffer and bound protein either eluted with increasing amounts of salt or SDS-PAGE sample buffer. For Pro-peptide affinity chromatography, the respective peptide was immobilized on CNBr-activated sepharose (Pharmacia) as recommended by the manufacturer.

Northwestern blotting and ssDNA agarose chromatography.

After binding of p65 from Jurkat cell lysates to a GST-*vav*SH2/SH3 matrix and eluting as described, fractions were run on SDS-PAGE, transferred to nitrocellulose and incubated with 50.000 cpm g-^{32}P-ATP labeled poly-rC/ml TBST-gelatin. After washing the filters, bound RNA was visualized by autoradiography. ssDNA-binding assays were done as described for hnRNP-K (Matunis et al., *Mol. Cell. Biol.* 12:164-171, 1992), here with Jurkat cell lysates.

It was determined that p65 is identical to hnRNP-K. Northwestern blot of a vavSH2/SH3 affinity matrix binding fractions was obtained. The matrix supernatant (S), protein eluting with between 300 mM and 1 M KCl (Lanes 1-4), and matrix bound protein that was eluted with SDS-sample buffer (B) was resolved by SDS-PAGE, blotted on NC membrane, and incubated with g-^{32}P-ATP labeled poly-rC. Also, ssDNA agarose binding assay of p65 was performed. Total lysates (L) that were bound to ssDNA-agarose, wash fraction after binding and matrix bound protein (B) was assayed by Far Western blotting with biotinylated GST-vavSH2/SH3-C fusion protein and in parallel by Western blotting with anti-hnRNP-K mAb B4B6.

vav mutagenesis.

Mutagenesis was performed as previously

described (16) with the following oligonucleotides for the PL834L mutant, 5'-CAC GTA GTT GGC AAG GAA CCA GCC AAC-3' (SEQ. I.D. NO. 3), and for the Y837F mutant 5'-ATC TTC CTC CAC GAA GTT GGC AGG GAA C-3'(SEQ. I.D. NO. 4). Each mutant was checked by standard DNA sequencing procedures.

Expression of vav in 293 embryonic kidney fibroblast.

Vav and respective mutants were cloned in pRK5 and transiently expressed in 293 cells which were subsequently analyzed as previously described (Lammers et al., *J. Biol. Chem.* 268:22456-22462, 1993).

Antibodies, Western Blotting and Immunoprecipitation.

The antibodies used for Western Blotting include a vav polyclonal, affinity purified antiserum raised against the GST-vavCysSH3-N fusion protein diluted 1:1000 in TBST-gelatin and monoclonal hnRNP-K antibody B4B6 (Dejgaard et al., *J. Mol. Biol.* 236:33-48, 1994) diluted 1:20. For immunoprecipitation, 1 µl of monoclonal hnRNP-K antibody 3C2 (Matunis et al., *Mol. Cell. Biol.* 12:164-171, 1992) was bound to 40 µl Protein-A Sepharose and incubated with the respective cell lysates for at least 3h. Beads were washed extensively with HNTG buffer and bound protein eluted with SDS-PAGE sample buffer.

An in vivo association of vav with hnRNP-K was observed. anti-hnRNP-K immunoprecipitates obtained from 293 cells expressing either wild type (wt) or mutated (YF for Y837F and PL for P834L) vav were checked for the presence of vav by Western blotting with an affinity-purified anti-vav polyclonal serum. Equal expression of hnRNP-K was checked independently.

Example 1: Association of PLC-γ with Polypeptides from 293 Extracts

In order to gain further insight into the function of PLCγ within the context of the pleiotropic responses of cells to growth factor stimulation, we

investigated the identity of proteins binding to the SH3 domain of this enzyme. To minimize potential conformational disturbance of the SH3 domain, we used a GST-SH2-SH2-SH3 domain fusion of PLCγ bound to immunoprecipitated RTKs to examine in vitro association with polypeptides from metabolically labelled 293 cell extracts.

Addition of increasing amounts of fusion protein to equal amounts of unlabelled immunoprecipitated human EGF-R, a human EGF/HER2 chimeric receptor, HER1-2 (Seedorf et al., *J. Biol. Chem.*, 266:12424-12431, 1991), and an EGF/PDGF-R chimera, EP-R (Aebersold et al., *Proc. Natl. Acad. Sci. USA* 84:6970-6974, 1987) caused, upon addition of [^{35}S]methionine-labelled cell extract, a parallel increase in binding of cellular proteins with apparent molecular weights of 110, 80, 65, and 43 kD, in the representative example of the HER1-2 chimera. (Seedorf et al., *J. Biol. Chem.*, 266:12424-12431, 1991).

The interaction of metabolically labeled proteins with HER1-2-bound GST-PLCγ-SH2-SH2-SH3 fusion protein was studied as follows. The HER1-2 receptor was transiently overexpressed in 293 fibroblasts and isolated by immunoprecipitation using 108.1 antibodies. Subsequently, Triton X-100 extract of [^{35}S]methionine labeled 293 cells was added in the absence, in the presence of decreasing amounts of GST-PLCγ-SH2-SH2-SH3 fusion protein (100 μg; 10 μg; 1 μg; 100 ng), or in the presence of 100 mg GST) and incubated for two hours. The immunocomplexes were washed, separated by SDS-PAGE, and analyzed by autoradiography. Protein bands that bind independent of increasing amounts of GST-PLCγ-SH2-SH2-SH3 fusion protein were marked. Part of the same samples were transferred to nitrocellulose membrane and probed with GST-specific antibodies. Protein bands were detected using horseradish peroxidase-coupled second antibody and ECL (Amersham) detection assay and included a 10 sec. exposure.

With the exception of small amounts of p110, these proteins were not detectable in the absence of the fusion protein, indicating that binding was largely mediated by the receptor-bound GST-PLCγ-SH2-SH2-SH3 protein. Moreover, since the SH2 domains were presumably engaged in interaction with receptor phosphotyrosine residues, the polypeptides detected in our association experiment may have bound to the PLCγ SH3 domain. To substantiate this, and to determine the identity of the binding proteins, we utilized a GST-PLCγ-SH3 fusion protein bound to glutathione sepharose to purify the major 110 kD binding protein from 293 cell extracts.

Fragmentation of the nitrocellulose-bound purified protein with Lys C endoproteinase and partial amino acid sequence determination by gas phase Edman degradation yielded the sequences (K)DYRQLELAXETQFEVDS (SEQ. I.D. NO. 12) and (K)TIMHLMINNT (SEQ. I.D. NO. 13). Comparison with the sequences in the Genbank database, using the search algorithm FASTA, revealed almost complete identity to the rat microtubule-activated nucleotide triphosphatase dynamin (Obar et al., *Nature* 347:256-261, 1990) at amino acid sequence positions 598-615 and 683-693.

Example 2: In Vitro Binding: 293 Fibroblasts

The SH3 binding activity of dynamin was confirmed by in vitro binding experiments with extracts of [^{35}S]methionine-labeled 293 fibroblasts that had been transfected with a human dynamin cDNA expression plasmid. (van der Bliek et al., *J. Cell Biol.* 122:553-563, 1993).

Binding of dynamin to glutathione sepharose-bound PLCγSH3 fusion protein was observed as follows. In *E. coli* expressed and purified GST, GST-PLCγ-SH2-SH2-SH3, GST-PLCγg-SH2-SH2, and GST-PLCγg-SH3 fusion protein was bound to glutathione sepharose as indicated and incubated with metabolically

[^{35}S]methionine labeled extracts from untransfected 293 cells and transfected 293 cells overexpressing human dynamin, respectively. The samples were washed with washing buffer, separated on a 7.5% SDS-polyacrylamide gel, and exposed to X-ray film. In parallel, aliquots of the same samples were analyzed by Western blot analysis using GST-specific antibodies. Protein bands were made visible using horseradish peroxidase-coupled second antibody and ECL (Amersham) detection assay.

Incubation with both transfected as well as untransfected 293 cell extracts led to the binding of a 110 kD protein to GST fusion proteins containing the SH3 domain either alone or as part of the 65 kD SH2-SH2-SH3 module, while no affinity for the 52 kD GST-SH2-SH2 fusion was detected. The intensity difference between untransfected and dynamin cDNA-transfected extracts identified the 110 kD band as dynamin, which was confirmed by immunoblot analysis with specific antibodies.

Example 3: Coimmunoprecipitation of PLC-γ, Dynamin, and EP-R Receptor

To demonstrate dynamin-PLCγ interaction in intact cells and to investigate the effect of RTK involvement, we performed coimmunoprecipitation experiments under various conditions. Metabolically labeled cell lysates were prepared from untreated and EGF-treated 293 fibroblasts overexpressing PLCγ, dynamin, or the chimeric receptor EP-R and combinations of these proteins after transfection with respective expression plasmids.

PLCγ-dynamin interaction in intact cells was observed as follows. 293 cells were transiently transfected with PLCγ, EP-R , PLCγg and dynamin, EP-R, PLCγ and dynamin, EP-R and PLCγ, EP-R and dynamin, and dynamin expression plasmid. Cells were metabolically labeled with [^{35}S]methionine overnight in the presence of 0.5% fetal calf serum and subsequently stimulated with

EGF (100 ng/ml) for 10 minutes. Cells were lysed and PLCγ was immunoprecipitated using PLCγ-specific antibodies. The samples were separated by SDS-PAGE (7.5%) and exposed to X-ray film.

Immunoprecipitation with a PLCγ-specific antiserum revealed coimmunoprecipitation of the 110 kD band from lysates of cells transfected with a dynamin expression vector either in combination with PLCγ or EP-R and PLCγ, but not EP-R alone.

Coimmunoprecipitation by anti-PLCγ antibodies was not influenced by coexpression with EP-R nor by ligand stimulation of EP-R, which caused a presumably phosphorylation-induced shift of the PLCγ band. This indicated that PLCγ may be bound to dynamin prior to interaction with an RTK and could associate with the ligand-activated receptors as a preformed complex. This was confirmed by immunoprecipitation of PLCγ, which resulted not only in coimmunoprecipitation of dynamin, but also of the EP-receptor in a ligand-dependent manner, demonstrating that PLCγ is able to connect the activated RTK to dynamin through its SH2 and SH3 domains. Moreover, in analogous experiments, immunoprecipitation of EP-R with the 108.1 monoclonal antibody against the extracellular EGF-R domain of the chimera, which led to EGF-dependent coimmuno-precipitation of PLCγ and dynamin, confirmed the PLCγ connector role between the RTK and dynamin.

Example 4: Coprecipitation of Dynamin and Various Signalling Factors

We next investigated dynamin interaction with other SH3 and SH2 domain-containing signalling factors in intact cells by performing coprecipitation experiments on transfected cell lysates.

Coimmunoprecipitation of dynamin with various signal transduction components was observed as follows. 293 cells were transiently transfected with a human dynamin expression plasmid alone and cotransfected with

PLCγ, GAP, p85, SHC, GRB-2, and c-raf1 vectors. In addition, DynI and DynIII, two dynamin variants containing mutations within the GTP-binding motifs, were coexpressed with wild-type GRB-2, and wild-type dynamin was coexpressed with GRB-2 1B and GRB-2 4B, each of which contain mutations in one of their SH3 domains. 24 hours later, the cells were metabolically labeled for 18 hours and lysed, followed by immunoprecipitation with specific antibodies against PLCγ, GAP, p85, SHC, GRB-2, and Raf1. Coimmunoprecipitation of dynamin was monitored after separation on a 10% SDS-polyacrylamide gel and exposure to X-ray film for 24 hours.

The association analysis included, in addition to the positive standard PLCγ, the SH3 domain proteins GAP, the p85 subunit of PI-3-kinase, and GRB-2. GRB-2 has been shown to link the EGF-receptor to the GDP/GTP exchanger SOS by binding of its SH2 domain to receptor-phosphorylated tyrosine residues and binding of its SH3 domain to a proline-rich sequence in the C-terminus of SOS. (Chardin et al., *Science* 260:1138-1143, 1993; Egan et al., *Nature* 363:45-51, 1993; Rozakis-Adcock et al., *Nature* 363:83-85, 1993; Li et al., *Nature* 363:85-88, 1993).

Furthermore, we included the GRB-2-SH3 mutants, GRB-2 1B (P49L) and GRB-2 4B (G203R), corresponding to the sem5 alleles n1619 and n2195 (Clark et al., *Nature* 356:340-344, 1992), which impair vulval development of *C. elegans* in this association analysis. In addition, we investigated the interaction of wild type GRB-2 with two GTP binding-deficient mutants, dynamin I and II, carrying substitutions in positions 44 (K/A) and 206 (K/D) (van der Bliek et al., *J. Cell Biol.* 122:553-563, 1993), and included as negative controls the signal-transducing factors SHC and Raf, which lack SH3 domains.

Immunoprecipitation of the non-SH3-containing proteins SHC and Raf with specific antisera did not

cause enhanced coprecipitation of coexpressed dynamin. The same result was obtained for GAP and p85, while PLCγ and GRB-2 antibodies clearly coimmunoprecipitated dynamin. Point mutations within the SH3 domains of GRB-2 which have been shown to abolish sem5 and GRB-2 functions *in vivo*, significantly reduced the extent of dynamin coprecipitation, suggesting that both SH3 domains are required for the interaction. However, dynamin and proteins of 140, 120, and 60 kD that bound constitutively to GRB-2, both in the presence and absence of growth factor stimulation, did not bind to the SH3 domains expressed individually, but only to a construct containing the N-terminal SH3 and the SH2 domain.

This indicates that in the case of GRB-2, only one of the SH3 domains is involved in domain binding and that the SH2 domain either participates directly or is required for the stabilization of the SH3 interaction. Mutations within the dynamin GTP-binding motif (dyn I and dyn III) did not affect its capability to form a physical complex with GRB-2, indicating that this function is dispensable for binding.

Example 5: Dynamin Binding to Fusion Proteins

We also investigated binding of dynamin to bacterially expressed GST-GRB-2, -c-crk, -PLCγ-SH3, -PLCγ-2SH2, -Nck, -p85-SH3, and -GAP in vitro. These fusion proteins were immobilized on glutathione-sepharose beads and incubated with extracts from PC12 rat pheochromocytoma cells. After elution and SDS-PAGE, dynamin binding was monitored by immunoblotting with specific antibodies.

Binding of dynamin to different GST-SH3 domain-containing fusion proteins was observed as follows. Bacterially expressed GST-SH3 domain-containing fusion proteins (3 μg) were immobilized on glutathione-sepharose, washed, and incubated with PC12 cell lysates (1 mg). The beads were washed 8 times with

RIPA buffer, heated in Laemmli's sample buffer, separated on 10% SDS-PAGE, and immunoblotted with anti-dynamin antibodies. Immunoblots were labeled with ^{125}I-protein A, followed by autoradiography.

Dynamin interacted under identical experimental conditions strongly with GST-GRB-2, at significantly lower affinity with GST-PLCγ-SH3, and very weakly with GST-p85-SH3, but not with the GST control, GST-c-crk, GST-PLCγ-2SH2, GST-Nck, or GST-GAP.

Recently, the tertiary structure of the SH3 domains for spectrin (Musacchio et al., *Nature* 359:851-855, 1992), p85 (Booker et al., *Cell* 73:813-822, 1993; Koyama et al., *Cell* 72:945-952, 1993), src (Yu et al., *Science* 258:1665-1668,1992), and PLCγ (Kohda et al., *Cell* 72:953-960, 1993) have been determined and shown to feature the same basic fold consisting of two approximately orthogonal β-sheets of three polypeptide strands. The conserved SH3 residues are in close proximity and form a hydrophobic patch on the surface which is thought to mediate the binding to proline-rich sequences. Such motifs have been identified in 3BP-1 and SOS and were shown to be the binding target for SH3 domains of abl and GRB-2, respectively. (Rozakis-Adcock et al. *Nature* 363:83-85, 1993; Ren et al. *Science* 259:1157-1161, 1993). Mutational analysis of the 3BP-1 SH3 binding motif and sequence comparison with other SH3 binding proteins such as formin and the muscarinic acethylcholine receptor revealed a possible binding consensus motif, XPXXPPPXXP (SEQ. I.D. NO. 14). (Cicchetti et al., *Science* 257:803-806, 1992; Ren et al., *Science* 259:1157-1161, 1993) A sequence matching these criteria, DPFGPPPQVP (SEQ. I.D. NO. 15), is located at position 825-833, while another proline-rich motif, PPVPSR (SEQ. I.D. NO. 16), which resembles the recently described SOS-GRB-2-SH3 interaction site (PPVPPR) (SEQ. I.D. NO. 17) (Rozakis-Adcock et al., *Nature* 363:83-85, 1993) is found between amino

acids 813 and 818.

Example 6: Peptide Competition Experiments

To determine whether this site or other proline-rich sequences found in dynamin are involved in the binding to the PLCγ-SH3 domain, four dynamin sequences were selected for peptide competition experiments:

peptide 1: APAVPPARPG (aa 785-794) (SEQ. I.D. NO. 18);
peptide 2: GPAPGPPPAG (aa 797-806) (SEQ. I.D. NO. 19);
peptide 3: APPVPSRPG (aa 812-820); (SEQ. I.D. NO. 20); and
peptide 4: PFGPPPQVPS (aa 825-834) (SEQ. I.D. NO. 21).

Mapping of the dynamin region that mediates binding to the PLCγ-SH3 domain was performed as follows. Bacterial expressed GST-PLCγ-SH3 fusion protein was immobilized on glutathione sepharose, washed, and incubated with metabolically labeled 293 cell extracts containing overexpressed dynamin in the absence of peptide (-) or presence of decreasing peptide concentrations: 1 mM (1), 100 μM (2), 10 μM (3). The peptides used correspond to proline-rich sequences in the carboxy terminal region of dynamin. The beads were washed with washing buffer, separated on a 10% SDS-polyacrylamide gel, and exposed to X-ray film for 36 hours.

Peptide 3 inhibited dynamin binding to the glutathionine-sepharose-bound PLCγ-SH3 domain at a concentration of 1 mM, while peptide 2 had no effect and peptides 1 and 4 only a minor effect. The same result was obtained in a similar experiment with the complete immunoprecipitated PLCγ protein, indicating that peptide 3 corresponded to a dynamin binding site.

In order to determine whether GRB-2 binds via its SH3 domains to the same proline-rich sequence, a GST-fusion protein with the dynamin amino acid sequence fragment 791-819 (ARPGSRGPAPGPPPAGSALGGAPPVPSR) (SEQ. I.D. NO. 22) was expressed in bacteria and after

SDS-PAGE, transferred to nitrocellulose followed by an overlay with GST-GRB-2 solution.

Identification of a dynamin proline-rich region that mediates binding to GRB-2 was performed as follows. GST-dynamin fusion protein corresponding to a proline-rich region, ARPGSRGPAPGPPPAGSALGGAPPVPSR (SEQ. I.D. NO. 23) (amino acids 791-819), was expressed in bacteria following transformation with a corresponding double-stranded cDNA fragment constructed from complementary oligonucleotides synthesized according to the coding and noncoding strands of the desired DNA sequence and engineered to contain the appropriate restriction sites. This was fused in-frame to the GST gene in pGEX2T. Lysates of GST-dynamin proline-rich peptide and GST-expressing bacteria were run on SDS-PAGE, transferred to nitrocellulose, followed by an overlay with GST-GRB-2 peptide solution. Bound GST-GRB-2 fusion protein was made visible using αGRB-2 antibodies and ^{125}I-protein A, followed by autoradiography.

Probing with GRB-2-specific antibodies resulted in detection of the GST-dynamin fusion protein, but not the GST control.

Example 7: Protein Binding to vav Subdomains

To investigate the signal transduction potential of vav, we characterized the interaction of different subdomains with proteins in lysates of cells in which vav appears to be involved in the regulation of cellular responses to external stimuli. For this purpose, Jurkat cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and analyzed using biotinylated GST-vav domain fusion proteins as probes. These contained either the vav C-terminal SH2 and SH3 domains, vavSH2/SH3-C, the N-terminal domain, N-vav, whose deletion renders vav oncogenic, or a central domain including the cysteine-rich motif (Adams

et al., *Oncogene* 7:611-618, 1992) and the second SH3 domain, vavCysSH3-N (Fig. 1). As a control, blots were incubated with labeled GST protein alone. Protein binding was visualized by incubation with streptavidin-coupled alkaline phosphatase and appropriate substrates.

It was observed that a 65 kD protein bound specifically to the GST-vavSH2/SH3-C fusion protein, since no binding to GST-N-vav, GST-vavCysSH3-N, or GST alone could be detected. Using the same probes, p65 was not only detected in both cytosol and nuclear extracts of a variety of haematopoietic cells, including Jurkat, KG-1, K562, MOLT15, and HEL, but also in non-haematopoietic 293 human embryonic fibroblasts.

Example 8: Binding of Native p65 to vav subdomains

To demonstrate that the vav domain fusion protein also bound the native form of p65 in solution, Sepharose beads carrying immobilized GST-vavSH2/SH3-C were incubated with cytosolic Jurkat cell extracts followed by elution with a [KCl] step gradient.

As shown by Coomassie staining of gel electrophoretically analyzed fractions, the only protein that bound to the affinity matrix eluted at 1 M KCl and had an apparent molecular weight of 65,000, while no protein was found to bind to a GST control affinity matrix. Its identity as the previously observed vav SH2/SH3 binding protein was confirmed by Far Western blot analysis with the GST-vavSH2/SH3-C probe.

Example 9: Identification of p65 as hnRNP-K

For partial amino acid sequence analysis, p65 was purified from crude lysates of Jurkat cells by vavSH2/SH3 affinity chromatography followed by tryptic digestion and separation of peptides by HPLC. Partial amino acid sequences were determined (Table 1) by Edman degradation using a gas phase sequencer.

Comparison of the amino acid sequences with the GenEMBL protein sequence database revealed that p65 was

identical to the heterogeneous ribonucleoprotein K, hnRNP-K (Matunis et al., *Mol. Cell. Biol.* 12:164-171, 1992; Table 1), a protein that had previously been identified in hnRNP-particles (Pinol-Roma et al., *Genes & Dev.* 2:215-227, 1988), and whose expression has been shown to be upregulated in transformed keratinocytes (Dejgaard et al., *J. Mol. Biol.* 236:33-48, 1994). The same protein had recently been shown to have transcription factor activity on the *c-myc* gene CT-promoter element (Takimoto et al., *J. Biol. Chem.* 268:18249-18258, 1993).

To confirm this finding and to examine whether the p65 vavSH2/SH3 binding protein actually exhibits properties of a ribonucleoprotein, we tested its RNA binding and ssDNA binding capacity. North Western blot probing of the *vav*SH2/SH3 matrix binding fraction with ^{32}P-labeled poly-rC and Far Western blot analysis of proteins isolated by ssDNA-Agarose chromatography from Jurkat cell lysates with labeled vavSH2/SH3 confirmed p65 as an hnRNP. Moreover, the ssDNA-agarose bound 65 kD protein was confirmed to represent hnRNP-K by Western blot analysis of the ssDNA-Agarose binding fraction with anti hnRNP-K mAb. Taken together, peptide sequence analysis, immunological cross reactivity, nucleic acid binding characteristics, and molecular weight demonstrated that the vavSH2/SH3 binding protein was identical to hnRNP-K.

Example 10: hnRNP-K Binding to SH2 and SH3 domains

To further investigate hnRNP-K binding characteristics, we constructed GST-fusion proteins with either vav SH2 or SH3 domains and, after biotinylation, used them in Far Western blot analysis experiments on crude Jurkat cell extracts.

While no binding to the SH2 domain was detected, the C-terminal SH3 domain was sufficient to bind hnRNP-K, albeit with lower affinity than the SH2/SH3 domain fusion. The binding of hnRNP-K to the

vav C-terminal SH3 domain was specific, since neither the N-terminal *vav* SH3 domain detected hnRNP-K in Far Western blots nor was any binding of hnRNP-K to the SH3 domains of PLCg or full-length GRB-2 containing two SH3 and one SH2 domain (Clark et al., *Nature* 356:340-344, 1992; Lowenstein et al., *Cell* 70:431-442, 1992) detectable.

Far Western blot of Jurkat extracts with biotinylated GST-vavSH2/SH3, GST-vavSH3 and GST-vavSH2 was obtained. hnRNP-K binds specifically to vavSH2/SH3-C. Affinity chromatography was performed with glutathione-sepharose immobilized GST-vavSH2/SH3-C, GST-GRB-2 and GST-PLCg-SH3 fusion protein. Matrix bound protein was checked for the presence of hnRNP-K by Western blotting with anti-hnRNP-K mAb B4B6. hnRNP-K binding to a GST-vavSH2/SH3 wild type affinity matrix, GST-vavSH2/SH3 Y837F affinity matrix and GST-vavSH2/SH3 P834L affinity matrix was obtained. Non-bound protein in the supernatant (S) and matrix bound protein (B) were assayed for the presence of hnRNP-K by Western Blotting with anti-hnRNP-K mAb B4B6.

The hnRNP-K Pro-rich motif is sufficient to bind vav. Total Jurkat cell lysates, the flow-through of the Pro-motif Sepharose matrix and a sepharose control matrix, a wash fraction and protein eluting at increasing amounts of KCl from the respective matrices were checked for the presence of vav by Western Blotting with an affinity-purified anti-vav polyclonal serum.

Example 11: hnRNP-K Binding to SH3 Domains With Point Mutation

Further confirmation of the binding of hnRNP-K to the SH3 domain of vav was obtained by introduction of a point mutation at position 834 of the C-terminal vav SH3 domain, which substituted a proline residue with a leucine. This mutation was analogous to a SH3 mutation found to abrogate the function of the GRB2 homolog *sem5* in the vulva differentiation pathway of *C. elegans*

(Clark et al., *Nature* 356:340-344, 1992; Lowenstein et al., *Cell* 70:431-442, 1992). The vavSH3-C P834L and a vavSH3-C Y837F mutant were expressed in *E. coli* as GST-fusion proteins in the context of the vavSH2/SH3 fragment. Equal amounts of wild-type and mutant proteins were immobilized on glutathione sepharose beads and examined for their ability to bind hnRNP-K from Jurkat cell lysates.

The P834L mutation completely abolished hnRNP-K binding, while the Y837F mutation had only a minor effect, further suggesting the C-terminal SH3 domain of vav as the binding site for hnRNP-K.

Example 12: Binding of vav to a Pro-Motif Matrix

An additional indication of the role of hnRNP-K as a SH3-domain binding protein was the existence of a proline-rich sequence stretch, PLPPPPPPRG (SEQ. I.D. NO. 5), in its C-terminal region, which differs from other previously identified SH3 binding motifs (Ren et al., *Science* 259:1157-1161, 1993; Yu et al., *Cell* 76:933-945, 1994). To determine whether this proline-rich sequence is involved in binding to *vav*, a peptide with the sequence RPLPPPPPPRGR (SEQ. I.D. NO. 6) was covalently bound to CNBr-activated sepharose and subsequently incubated with Jurkat cell extracts. In contrast to control Sepharose, RPLPPPPPPRGR-Sepharose (SEQ. I.D. NO. 7) beads specifically bound $p95^{vav}$ from the Jurkat cell lysates.

Example 13: Interaction of Ectopically Expressed vav and nhRNP-K in Intact Cells

To investigate the interaction of vav and hnRNP-K in the context of a living cell, we ectopically expressed vav and the two SH3 mutants in 293 human embryonic fibroblasts. This approach avoided interference by endogenously expressed vav and therefore allowed examination of the effects of SH3 domain mutants on the interaction with endogenous hnRNP-K.

Anti-hnRNP-K immunoprecipitates were found to

contain readily detectable amounts of vav protein in cells transfected with a wild type vav expression vector. In contrast, the P834L mutant did not coimmunoprecipitate with hnRNP-K, while the Y837F mutation caused only a minor decrease on binding affinity. These data not only confirmed the results of our *in vitro* binding experiments, but also clearly demonstrated that ectopically expressed vav is able to interact with endogenous hnRNP-K in intact 293 cells.

Other embodiments are within the following claims.

Table 1.
Peptide sequences derived from purified, proteolytically cleaved p65.

p65 sequence	Matching sequence in hnRNP-K (amino acid positions)
DEMVELRILLQS (SEQ. I.D. NO. 10)	40-51
KIILDLISESPIK (SEQ. I.D. NO. 11)	207-219
DLGXP (SEQ. I.D. NO. 12)	382-386
DQIQNAQYLLQNS (SEQ. I.D. NO. 13)	442-454

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Axel Ullrich and Günter Kostka

(ii) TITLE OF INVENTION: METHODS FOR TREATMENT OR DIAGNOSIS OF DISEASES OR CONDITIONS ASSOCIATED WITH A DYN DOMAIN

(iii) NUMBER OF SEQUENCES: 23

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(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM compatible
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: to be issued
(B) FILING DATE: May 31, 1994
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

Prior application total, including application described below: none

(A) APPLICATION NAME:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: X_{aa} in positions 2, 3, 4, 7 and 8 is any amino acid.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro X_{aa} X_{aa} X_{aa} Pro Pro X_{aa} X_{aa} Pro
5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: X_{aa} in positions 2, 3, 6 and 7 is any amino acid.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Pro X_{aa} X_{aa} Pro Pro X_{aa} X_{aa} Pro
5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 3:

Ala Pro Pro Val Pro Ser Arg Gly
5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 4:

Ala Pro Pro Val Pro Ser Arg Pro Gly
5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 5:

Pro Pro Val Pro Pro Arg
5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 6:

Ala Pro Pro Val Pro Ser Arg
5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 7:

Ala Pro Ala Val Pro Pro Ala Arg Pro Gly
5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 8:

Pro Phe Gly Pro Pro Pro Gln Val Pro Ser
5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 9:

Ala Pro Pro Val Pro Ser Arg Pro Gly
5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 10:

Ala Pro Ala Val Pro Pro Ala Arg Pro Gly
5 10

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Pro Phe Gly Pro Pro Pro Gln Val Pro Ser
5 10

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) FEATURE:

(D) OTHER INFORMATION: X_{aa} in position 10 is any amino acid.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Lys Asp Tyr Arg Gln Leu Glu Leu Ala X_{aa} Glu Thr Gln Phe Glu Val
5 10 15

Asp Ser

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 13:

Lys Thr Ile Met His Leu Met Ile Asn Asn Thr
5 10

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: X_{aa} in positions 1, 3, 4, 8, and 9 is any amino acid.

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 14:

X_{aa} Pro X_{aa} X_{aa} Pro Pro Pro X_{aa} X_{aa} Pro
5 10

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 15:

Asp Pro Phe Gly Pro Pro Pro Gln Val Pro
5 10

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 16:

Pro Pro Val Pro Ser Arg
5

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 17:

Pro Pro Val Pro Pro Arg
5

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 18:

Ala Pro Ala Ver Pro Pro Ala Arg Pro Gly
5 10

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 19:

Gly Pro Ala Pro Gly Pro Pro Pro Ala Gly
5 10

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Ala Pro Pro Val Pro Ser Arg Pro Gly
5

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Pro Phe Gly Pro Pro Pro Gln Val Pro Ser
5 10

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Ala Arg Pro Gly Ser Arg Gly Pro Ala Pro Gly Pro Pro Pro
5 10

Arg Gly Ser Ala Leu Gly Gly Ala Pro Pro Val Pro Ser Arg
20 25

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Ala Arg Pro Gly Ser Arg Gly Pro Ala Pro Gly Pro Pro Pro
5 10

Ala Gly Ser Ala Leu Gly Gly Ala Pro Pro Val Pro Ser ARg
15

Other embodiments are within the following claims.

Claims

1. Method for treatment of a disease or condition in an organism, characterized by an abnormality in a signal transduction pathway, said signal transduction pathway involving the interaction between;

(a) a receptor tyrosine kinase of the Trk family and a signaling component;

(b) a heterogenous ribonucleoprotein MP domain and a SH3 domain;

(c) a MP domain and a vav protein SH3 domain; or

(d) a SH3 domain and a DYN domain

comprising the step of disrupting or promoting said interaction in vivo.

2. Method for screening for an agent useful for treatment of a disease or condition characterized by an abnormality in a signal transduction pathway, said signal transduction pathway involving the interaction between;

(a) a receptor tyrosine kinase of the Trk family and a signaling component;

(b) a heterogenous ribonucleoprotein MP domain and a SH3 domain;

(c) a MP domain and a vav protein SH3 domain; or

(d) a SH3 domain and a DYN domain

comprising assaying potential said agents for those able to disrupt or promote said interaction as an indication of a useful said agent.

3. Method for diagnosis of a disease or condition characterized by an abnormality in a signal transduction pathway, said signal transduction pathway involving the interaction between;

(a) a receptor tyrosine kinase of the Trk family and a signaling component;

(b) a heterogenous ribonucleoprotein MP domain and a SH3 domain;

(c) a MP domain and a vav protein SH3 domain; or

(d) a SH3 domain and a DYN domain

comprising the step of detecting the level of said interaction as an indication of said disease or condition.

4. The method of any of claims 1-3 wherein said disease or condition is selected from the group consisting of neurodegenerative disorders, neuroproliferative disorders or cancer.

5. The method of any of claims 1-3 wherein said receptor tyrosine kinase of the Trk family is TrkA.

6. The method of any of claims 1-3 wherein said signalling component is SHC.

7. The method of any of claims 1-3 wherein said heterogenous ribonucleoprotein is heterogenous ribonucleoprotein K.

8. The method of any of claims 1-3 wherein said heterogenous ribonucleoprotein is not p62.

9. The method of any of claims 1-3 wherein said heterogenous ribonucleoprotein MP domain is PLPPPPPPRG (SEQ. ID. NO. 8).

10. The method of any of claims 1-3 wherein said SH3 domain is not part of GAP.

11. The method of any of claims 1-3 wherein said SH3 domain is a PLC-γ or GRB-2 SH3 domain.

12. The method of any of claims 1-3 wherein said vav protein is $p95^{vav}$.

13. Method for screening non-haematopoietic cells for a protein having an SH3 domain that binds a heterogenous

ribonucleoprotein MP domain comprising the steps of exposing a protein having a SH3 domain from a non-haematopoietic cell to a heterogenous ribonucleoprotein MP domain and detecting the level of interaction between said domains.

14. The method of claim 13 wherein said heterogenous ribonucleoprotein MP domain is PLPPPPPPRG (SEQ. ID. NO. 9).

15. Peptide consisting essentially of a DYN domain.

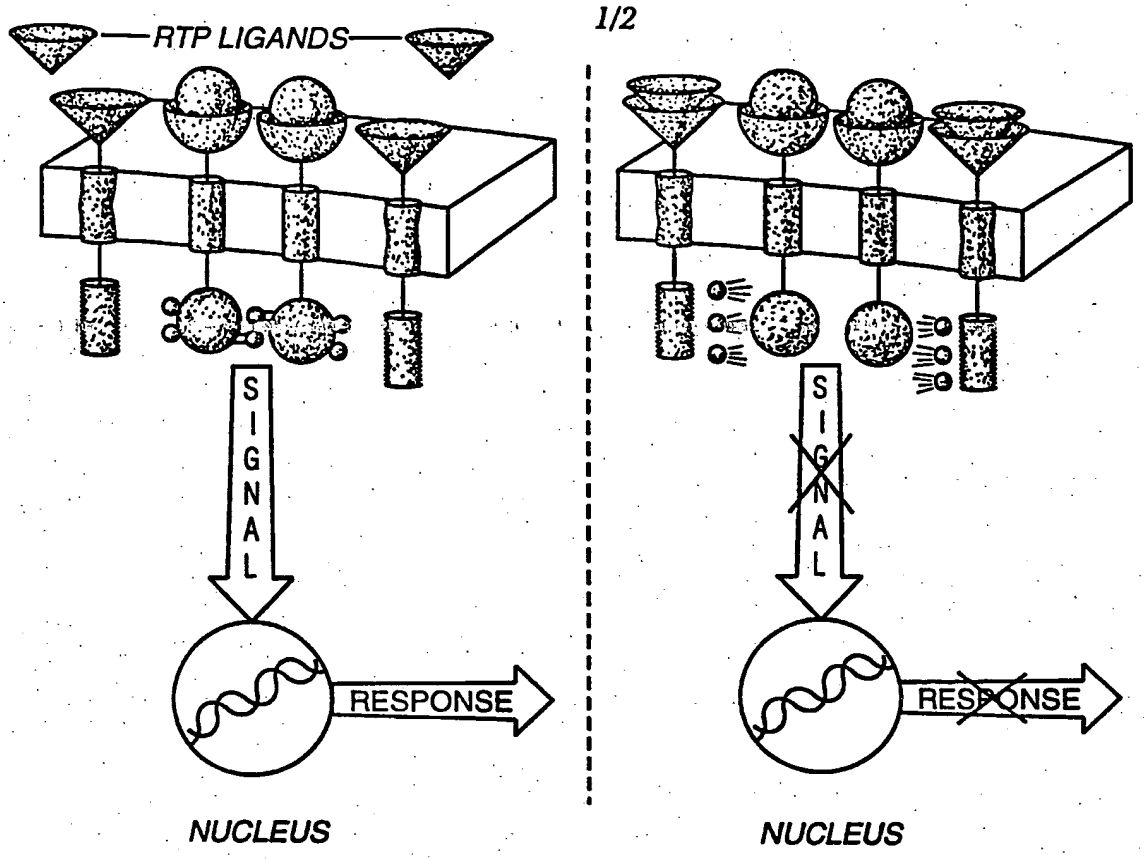
RTP LIGANDS
SIGNAL
RESPONSE
NUCLEUS
SIGNAL
RESPONSE
NUCLEUS

FIG. 1

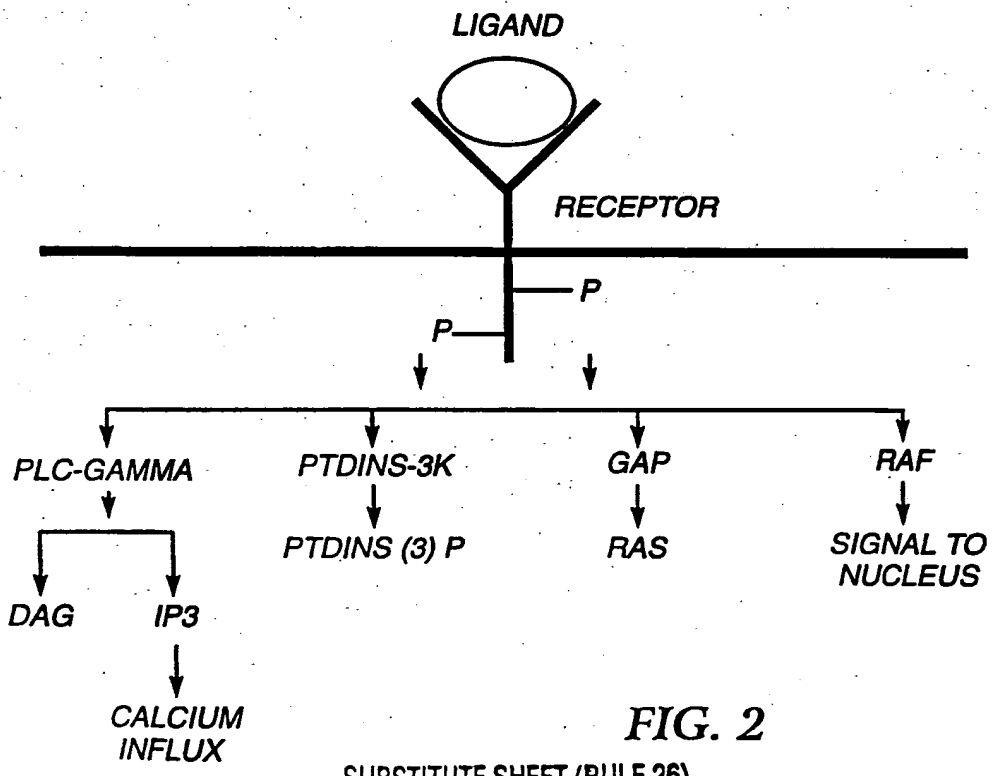
LIGAND
RECEPTOR
P
P
PLC-GAMMA
PTDINS-3K
GAP
RAF
DAG
IP3
PTDINS (3) P
RAS
SIGNAL TO NUCLEUS
CALCIUM INFLUX

FIG. 2

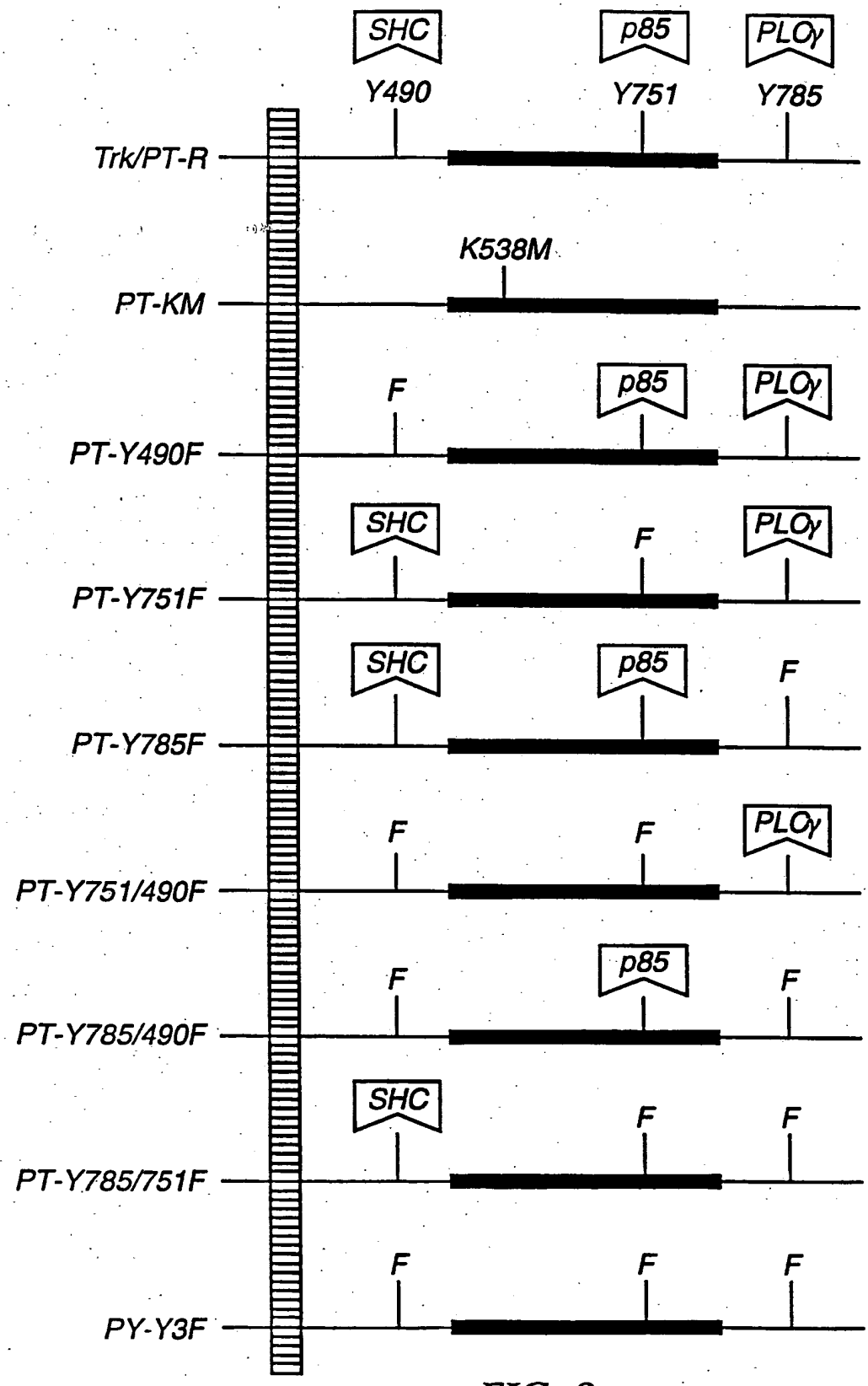
SHC
p85
PLCγ
Y490
Y751
Y785
Trk/PT-R
K538M
PT-KM
F
PT-Y490F
PT-Y751F
PT-Y785F
PT-Y751/490F
PT-Y785/490F
PT-Y785/751F
PY-Y3F

FIG. 3

ANY REFERENCE TO FIGURE 4 SHALL BE CONSIDERED NON-EXISTENT

(See Article 14(2))